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Astroglial Insulin-like Growth Factor Binding Proteins: Regulation Of Synthesis And Relationship To Cell Growth

Sheri Lynn Bradshaw

ASTROGLIAL INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS: REGULATION OF SYNTHESIS AND RELATIONSHIP TO CELL GROWTH

by

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- Civil 0543
- Electronics and Electrical 0544
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- Hydraulic 0545
- Industrial 0546
- Marine 0547
- Materials Science 0794
- Mechanical 0548
- Metallurgy 0743
- Mining 0551
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ABSTRACT

The insulin-like growth factors (IGFs) regulate the growth and differentiation of a wide variety of tissues, including the brain. The biologic actions of IGFs are modulated by a family of six IGF binding proteins (IGFBPs), which, like IGFs, are synthesized as paracrine factors. We hypothesized that the IGFBPs. synthesized by astroglial cells, are regulated by growth factors produced in the developing brain, and modulate the growth of these cells. Primary astroglial cells synthesized IGFBP-2 and IGFBP-3 which were upregulated in a dose dependent manner by IGF-I, IGF-II, and very high concentrations of insulin, suggesting that the growth factors regulate their own modulating IGFBPs. Transforming growth factor-α, epidermal growth factor, and acidic and basic fibroblast growth factors differentially regulated IGFBP biosynthesis suggesting interaction among growth factor systems via IGFBPs. Overexpression of IGFBP-2 in the C6 glioma cell line resulted in clones with variable growth rates. Clones secreting high levels of IGFBP-2 and IGF-I, maintained a similar growth rate, whereas those which expressed moderate levels of IGFBP-2 and low levels of IGF-I, had significantly reduced growth rates. The slow growing clones also had a membrane associated IGFBP which was deduced to be IGFBP-5. Exogenous addition of IGF-I accelerated growth rates of the wild type and the fast growing clones, but not those which had cell surface associated IGFBP-5, suggesting the importance of this IGFBP in cellular growth. Furthermore, C6 cells, which have a low level of expression of connexin43 synthesized high levels of IGF-I and negligible amounts of IGFBP-2. C6 cells overexpressing a connexin43 cDNA have reduced growth rates and expressed less IGF-I and different IGFBP profiles. These studies suggested that (i) astroglial IGFBPs are regulated by various

growth factors synthesized within the brain and (ii) growth of astroglial cells is regulated by a coordinated interaction between IGFs and IGFBPs, and specific properties of the latter.

Key words: Insulin-like growth factor, binding proteins, astroglia, regulation, growth

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TABLE OF CONTENTS

Page

CHAPTER FIVE:

ALTERATIONS IN THE SYNTHESIS OF IGFBPs AND IGFs IN C6 GLIOMA CELLS TRANSFECTED WITH A GAP JUNCTION CONNEXIN43 cDNA

Page

CHAPTER SIX:

C6 GLIOMA CELLS TRANSFECTED WITH AN IGFBP-2 cDNA: CHARACTERIZATION OF CLONES AND COMPARISON OF GROWTH

CHAPTER SEVEN:

DETECTION AND CHARACTERIZATION OF A CELL SURFACE ASSOCIATED IGFBP FOUND ON THE SLOW GROWING CLONES

Page

LIST OF FIGURES

Page

Page

Page

LIST OF TABLES

LIST OF APPENDICES

GENERAL ABBREVIATIONS

%	per cent
^{3}H	tritium
^{32}P	phosphorous-32
^{35}S	sulfur-35
^{125}I	iodine-125
BME	Basal Medium Eagle
BSA	bovine serum albumin
℃	degrees celsius
Ci	curie
cDNA	complementary DNA
cRNA	complementary RNA
CTP	cytidine 5'-triphosphate
d	day(s)
ddH20	double distilled water
DEPC	diethyl pyrocarbonate
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
DSS	disuccinimidyl suberate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N, N, N', N'-tetraacetic acid
FBS	fetal bovine serum
FGF	fibroblast growth factor
g	gram
g	acceleration of gravity
GFAP	glial fibrillary acidic protein
h	hour(s)
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
M	molar
min	minute(s)
mRNA	messenger RNA
n	nano
NaCl	sodium chloride
PBS	Phosphate buffered saline
RNA	ribonucleic acid
RNAse	ribonuclease

RT	room temperature
s	second(s)
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SFM	serum free medium
SSC	sodium citrate/sodium chloride solution
TBS	Tris buffered saline
TCA	trichloroacetic acid
TGF-α	transforming growth factor-alpha
Tris	hydroxymethyl aminomethane
TTBS	Tween-Tris buffered saline
w/v	weight per volume
μ	micro
v/v	volume per volume

CHAPTER ONE

GENERAL LITERATURE REVIEW

1.1 INSULIN-LIKE GROWTH FACTORS

1.1.1 Discovery

The insulin-like growth factors (IGFs) were first discovered by their ability to mediate the effects of growth hormone (GH) on long bone growth. Salmon and Daughaday (1957) determined that the incorporation of sulfate into cartilage, as a measure of bone growth, was impaired in hypophysectomized rats and could be restored by administration of GH. GH, or serum from hypophysectomized rats, were unable to stimulate sulfate incorporation of hypophysectomized cartilage *in vitro*, however, serum from normal rats was able to stimulate a two- to three-fold increase in sulphate uptake. They hypothesized that there was an intermediary substance in serum required for the action of GH. This substance, originally termed "sulphation factor", was found to have a wide range of biological activities including, stimulation of i) DNA synthesis (Daughaday and Reeder 1966), ii) proteoglycan synthesis (Hall and Uthne 1971), iii) glycosaminoglycan synthesis (Daughaday et al. 1975), and iv) protein synthesis (Salmon and Duvall 1970). The "sulphation factor" was renamed "somatomedin" (Daughaday et al. 1972), for being the mediator of the effects of somatotrophin (GH) and thus "the somatomedin hypothesis" was coined.

At the time when the mediator of GH was being identified, concurrent studies were ongoing to define serum factors that could stimulate insulin-like effects. These factors were known to be distinct from immunoreactive insulin, as their actions could not be abolished by the addition of specific anti-insulin antibodies, and were thus called non suppressible insulin-like activity (NSILA) (Froesch et al. 1966). Highly purified NSILA and somatomedin from serum extracts displayed similar biological activities of stimulating sulfate

incorporation into cartilage and stimulating glucose incorporation into fat. It was thought that these factors might be very similar to each other and to the structure of insulin. Eventual purification and amino acid sequencing of two bioactive NSILA factors from Cohn plasma protein fractions revealed homology to pro-insulin, and were thus designated insulin-like growth factor-I (Rinderknecht and Humbel 1978a) and insulin-like growth factor-II (Rinderknecht and Humbel 1978b).

In addition, other groups were investigating factors which sustained the growth of cultured cells in the absence of serum. NSILA purified from calf serum was found to be a potent stimulator of cell growth in culture (Pierson et al. 1972). This multiplication-stimulating activity was purified from a cell line that grew well in the absence of serum and was demonstrated to have growth promoting activity upon other cells in culture (Dulak and Temin 1973). Eventual purification and determination of its primary structure revealed that this rat peptide was nearly identical to the previously purified human IGF-II (Marquardt et al. 1981). These studies demonstrated that IGFs could be synthesized and act on the same cells, thus the autocrine/paracrine nature of IGFs was established.

Since this thesis deals with the role of IGFs and IGFBPs in the growth of rat astroglial cells, the following review of the IGF system will emphasize the biochemical and molecular structure of the rat components of the IGF system with some reference to other species as indicated.

1.1.2 Insulin-like growth factor-I

1.1.2.1 Structure

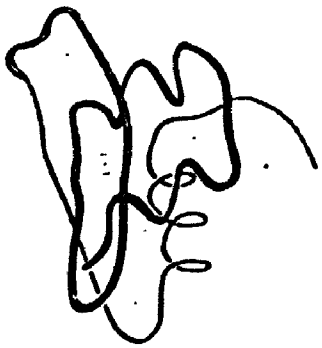
Molecular cloning of the human IGF-I cDNA confirmed the primary sequence of IGF-I first purified from human plasma (Jansen et al. 1983). Rat IGF-I is a 70 amino acid, basic, single chain polypeptide, with a predicted molecular weight of 7 649 daltons. Comparison of the primary sequence of IGF-I revealed that it shares 48% homology with proinsulin, is organized into similar B, C, and A domains, and contains a carboxyl-terminal extension (D domain) not found in proinsulin. Tertiary structure is maintained by three conserved di-sulphide bridges (Raschdorf et al. 1988, Smith et al. 1989). An amino-terminal signal peptide and a carboxyl-terminal E domain peptide are proteolytically cleaved from a pre-pro-IGF-I peptide to generate mature IGF-I. A three-dimensional model of the IGFs (Figure 1.1) was proposed by computer modeling using the X-ray structure of insulin and proinsulin as templates (Blundell et al. 1978, Blundell and Humbel 1980). Nuclear magnetic resonance analysis has confirmed the predicted structures for IGF-II (Terasawa et al. 1994).

A variant of IGF-I which lacks the first three amino acids has been purified and characterized from brain extracts and designated des(1-3)IGF-I (Sara et al. 1986, Carlsson-Skwirut et al. 1986). Removal of the first three amino acids reduces the affinity of IGF-I for their binding proteins (Forbes et al. 1988, Carlsson-Skwirut et al. 1989) which is thought to account for the increased potency of des(1-3)IGF-I both *in vitro* (Bagley et al. 1989, Ballard et al. 1987, Remacle-Bonnet et al. 1992) and *in vivo* (Gillespie et al 1990, Tomas et al. 1991, 1993). A protease capable of generating des(1-3)IGF-I from intact IGF-I has been isolated from rat serum (Yamamoto and Murphy 1994).

Figure 1.1 Schematic diagram of the three dimensional structure of IGF-I (**A**) and proinsulin (**B**). The predicted structure of IGF-I was derived from computer modeling comparisons with the X-ray crystallographic derived structure of insulin and proinsulin (adapted from Blundell and Humbel 1980).

A **IGF-I**

B **proinsulin**



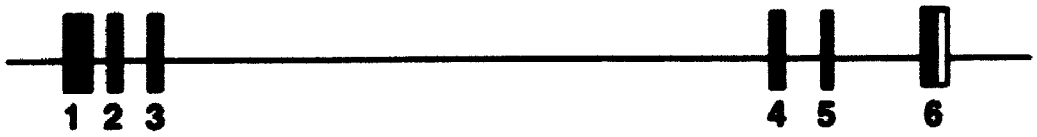
1.1.2.2 Gene structure

The rat IGF-I gene (Figure 1.2 A) spans more than 90 kb and contains at least 6 exons (Shimatsu and Rotwein 1987, Hall et al. 1992). The rat gene codes for two different pre-pro-IGF-I precursors by generating distinct mRNAs through alternative splicing, differential polyadenylation site usage, and the use of multiple promoters (Figure 1.2 B). IGF-Ia mRNAs are encoded by exons 1 or 2 and 3, 4, and 6 while IGF-Ib mRNAs are comprised of exons 1 or 2 and 3, 4, 5 and 6. The mature IGF-I peptide is encoded from sequences from exons 3 and 4. Exons 1 and 2 encode for different 5'-untranslated regions that have been shown to have multiple translation initiation sites (Roberts et al. 1987a). The 3' region of exon 4 and 5' region of exon 6 encode for the Ea domain peptide and the 3' region of exon 4, exon 5 and 5' region of 6 encode for the Eb domain peptide (Roberts et al. 1987b, Lowe et al. 1988). Exon 6 contains multiple polyadenylation sites (Hall et al. 1992).

The rat IGF-I gene contains two promoter regions, a major promoter which regulates transcription of mRNAs that contain exon 1, and a second promoter which regulates mRNAs containing exon 2 (Hall et al. 1992, Lowe et al. 1992, Adamo et al. 1993). The major promoter lacks both TATA and CAAT boxes, is not GC-rich, and multiple transcription initiation sites have been identified (Adamo et al. 1989, 1991). Expression of mRNAs from the major promoter occurs in all tissues. Analysis of the major promoter indicated that the basal activity of the promoter is localized to the region between nucleotides -18 and +78 of exon 1 and this region contains protein binding sites (Ra An and Lowe 1995). Expression of mRNAs generated from the minor promoter is restricted to the liver (Lowe et al. 1987, Hall et al. 1992). Additional promoter activity, including a repressor element, has been demonstrated 500 kb upstream of exon 1 (Huang et al. 1995).

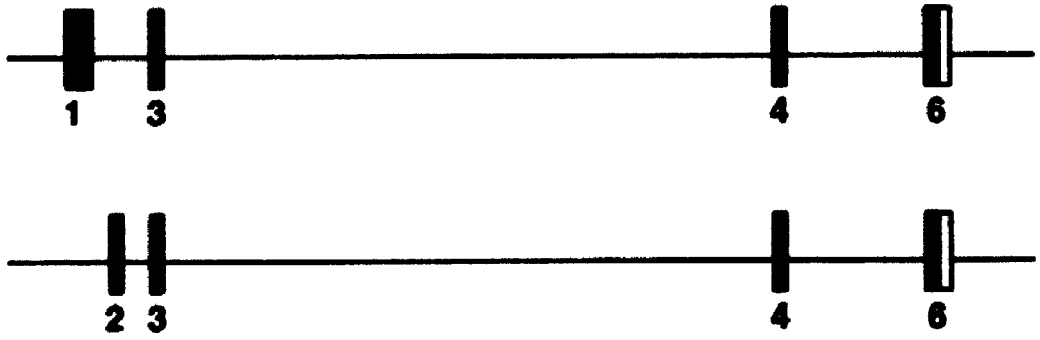
Figure 1.2 The organization of the rat IGF-I gene (**A**) and the exonic composition of the alternative IGF-Ia and IGF-Ib mRNA transcripts that are generated (**B**). Exons are represented by boxes, solid lines represent introns, and regions encoding pre-pro-IGF-I are shaded (adapted from Ward and Elliss 1992).

A Rat IGF-I gene structure

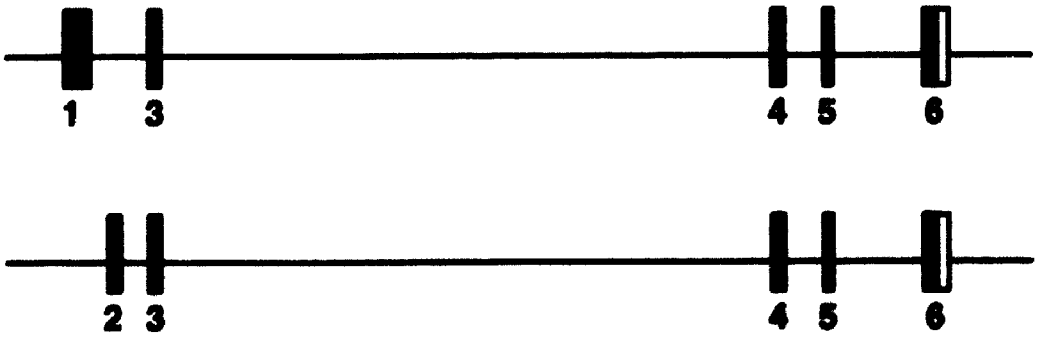


B The exonic composition of IGF-I mRNAs

IGF-Ia mRNAs



IGF-Ib mRNAs



Lund et al. (1989) have determined that the basis for the multiple mRNA transcript sizes in the liver is due primarily to differential polyadenylation site usage which generates IGF-I mRNA transcripts of 7.0-7.5 kb with long 3'-untranslated regions (UTR) and 1.2-0.9 kb with short 3'-UTR. Analysis of IGF-I mRNAs containing the long 3'-UTR, both *in vitro* and *in vivo*, demonstrated that they decayed much faster than the IGF-I mRNA transcripts with shorter 3'-UTR (Hepler et al. 1990). The *in vivo* translatability of the various mRNA transcripts has been analyzed by Foyt et al. (1991). They found that the various IGF-I mRNA transcripts which varied in their 5'-UTR sequences or their E-peptide coding sequences were all found associated with rat liver polysomal fractions. With respect to the variable 3'UTR of the IGF-I mRNAs, only the shorter transcripts were found associated with polysomes suggesting that some aspect of the long 3'-UTR affected translatability.

1.1.2.3 Regulation of expression

Growth hormone regulates circulating total IGF-I levels by increasing hepatic IGF-I gene expression and regulates tissue IGF-I levels by increasing specific tissue IGF-I gene expression (Mathews et al. 1986, Murphy et al. 1987, Roberts et al. 1987b, Hynes et al. 1987, Nilsson et al. 1990, Chin et al. 1992). Hypophysectomy reduces serum IGF-I (Glascock et al. 1990) and IGF-I gene expression in the liver, skeletal muscle, heart, white adipose tissue, kidney, spleen, and testes, but not brain (Gosteli-Peter et al. 1994). IGF-I mRNA levels can be restored in these tissues by GH infusion. IGF-I infusion can fully restore its level of expression in kidney, thymus and spleen, incompletely restore its expression in muscle and heart, has no effect on brain, testes, and white adipose tissue and decreases hepatic IGF-I mRNA levels (Schoenle et al.

1982, Skottner et al. 1989, Gosteli-Peter et al. 1994). Diabetes, fasting, and protein restriction reduce IGF-I gene expression (Emler and Schalch 1987, Lowe et al. 1989, Goldstein et al. 1988, Bornfelt et al. 1989, Clemmons and Underwood 1991).

In the fetus, IGF-I levels are independent of GH and are expressed by most tissues during development (Han et al. 1987a, Han et al. 1988). In the uterus, IGF-I expression is induced by estrogen independent of GH (Murphy et al. 1987, Norstedt et al. 1989).

1.1.2.4 Biological actions

IGF-I infusion in normal adult rats results in hypoglycemia by stimulating peripheral glucose uptake (Zapf et al. 1986, Jacob et al. 1989) and carcass weight gain (Thissen et al. 1991). In the neonatal rat, infusion of IGF-I also stimulates growth (Phillips et al. 1988). IGF-I has anabolic activity in a variety of catabolic states including fasting and dietary protein restriction (O'Sullivan et al. 1989, Thissen et al. 1991), and acute ischemic renal failure (Miller et al. 1992, Ding et al. 1993). IGF-I infusion in GH deficient and hypophysectomized adult rats stimulates body weight gain, tibial epiphyseal width and longitudinal bone growth (Schoenle et al. 1982, D'Ercole et al. 1984, Orlowski and Chernausek 1988, Guler et al. 1988, Skottner et al. 1989). In the diabetic rat, IGF-I stimulates organ and lean body weight without having effects on blood glucose and body fat (Scheiwiller et al. 1986, Jacob et al. 1991, Rosetti et al. 1991). IGF-I also stimulates wound healing (Lynch et al. 1989, Suh et al. 1992).

Studies on transgenic mouse models have also provided insights into the biological functions of IGF-I. GH transgenic mice show increased expression of endogenous IGF-I after the second to third week of postnatal life

resulting in accelerated postnatal growth with a two-fold increase in body weight (Palmiter et al. 1982, 1983, Mathews et al. 1988a). Mice overexpressing an IGF-I transgene exhibit a less dramatic increase in body weight which does not occur until 4 weeks postnatally (Mathews et al. 1988b). Total body weight gain was 30% greater than controls, and in contrast to GH transgenic mice, head to tail length and liver weight are not increased, and the brain has a dramatic 50% increase in weight. In the IGF-I transgenic animals, GH expression is suppressed by 60% resulting in a suppression of endogenous hepatic IGF-I expression. Hence, circulating IGF-I levels are elevated in the IGF-I transgenic mice but are significantly lower than in the GH transgenic mice possibly explaining the slower growth rate of the IGF-I transgenic mice. In order to dissect the effects of IGF-I transgene expression in the absence of GH, the IGF-I transgenic mice were bred with transgenic mice whose GH expression was ablated by expression of the diptheria toxin in somatotrophs (Behringer et al. 1988). Body weight and head to tail length of the IGF-I overexpressing, GH deficient mice are indistinguishable from control mice at all ages (Behringer et al. 1990). There is a disproportionate increase in brain weight and cell number in these transgenic mice, similar to the IGF-I transgenic mice, suggesting that IGF-I overexpression in the brain stimulates growth in an autocrine or paracrine manner.

Mice deficient in the IGF-I gene have reduced birthweights that are 60% compared to wild type littermates but maintain relative body proportions (Liu et al. 1993, Baker et al. 1993). These mice are infertile and have delayed bone development (Liu et al. 1993, Baker et al. 1993). Another group has produced mice lacking functional IGF-I which also display growth retardation and, in contrast to the other IGF-I null mice, most die at birth from respiratory failure (Powell-Braxton et al. 1993).

IGF-I has a wide variety of activities *in vitro* (Sara and Hall 1990, Jones and Clemmons 1995). IGF-I is a potent mitogen for numerous cell types including, fibroblasts (Rechler et al. 1974, Conover et al. 1983), astroglia (Han et al. 1987, Ballotti et al. 1987, Shemer et al. 1987), neurons (Shemer et al. 1987), chondrocytes (Froger-Gaillard et al. 1989, Bohme et al. 1992), osteoblasts (Hock et al. 1988), and others (reviewed in Jones and Clemmons 1995). IGF-I stimulates DNA synthesis and cell replication by functioning as a cell cycle progression factor which allows cells to progress through G1 and continue through the cell cycle (Pledger et al. 1977, 1978, Leof et al. 1982, Wharton 1983).

IGF-I has insulin-like metabolic activities in certain cell types in that it stimulates amino acid and glucose uptake and protein synthesis (Froesch et al. 1985). IGF-I promotes the differentiation of a number of cell types (Sara and Hall 1990, Jones and Clemmons 1995) including, myoblasts (Florini and Ewton 1990, 1991a), adipocytes (Smith et al. 1988), oligodendroglia (McMorris and Dubois-Dalq 1988), neurons (Pahlman et al. 1991), osteoblasts (Schmid et al. 1984), osteoclasts (Mochizuki et al. 1992) and others.

1.1.3 Insulin-like growth factor-II

1.1.3.1 Structure

The human, rat and mouse IGF-II genes each encode a 180 amino acid precursor, comprised of a 24 residue amino-terminal signal peptide, a 67 amino acid mature IGF-II, and a 89 amino acid carboxyl terminal pro-peptide. Mature IGF-II is proteolytically cleaved from the pre-pro-peptide and is a neutral peptide of Mr 7469 Da with 60% amino acid homology to IGF-I.

1.1.3.2 Gene structure

The rat IGF-II gene spans about 12 kb, contains 6 different exons, and multiple promoters which encode for multiple mRNA transcripts (Frunzio et al. 1986, Soares et al. 1986, Yamamoto 1990). Exons 1, 2, and 3 are non-coding and are differentially used to generate alternative 5'-untranslated regions of different mRNAs. Exons 4, 5, and 6 encode the pre-pro-IGF-II protein (Figure 1.3).

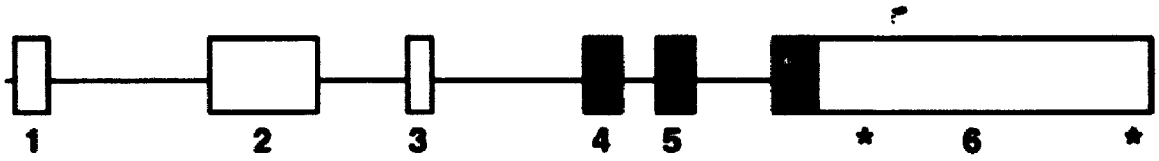
The rat IGF-II gene contains 4 promoter regions upstream of each of exons 1, 2, and 3 designated P1, P2, and P3, respectively (Frunzio et al. 1986, Soares et al. 1986, Ueno et al. 1987). Consensus TATA and GC boxes are present in P2 and P3. The GC boxes within P2 and P3 can bind the transcription factor Sp1 (Evans et al. 1988, Matsuguchi et al. 1990). The P1 promoter lacks TATA and GC boxes and has multiple transcription start sites (Ueno et al. 1987). The mRNA transcripts generated are approximately 3.8, 4.6, and 3.6 kb from promoters 1, 2 and 3 respectively. Transcripts derived from P2 and P3 are the most predominant while transcripts from P1 and multiple polyadenylation signals in exon 4 contribute to the other less abundant transcripts (Ueno et al. 1989).

1.1.3.3 Regulation of expression

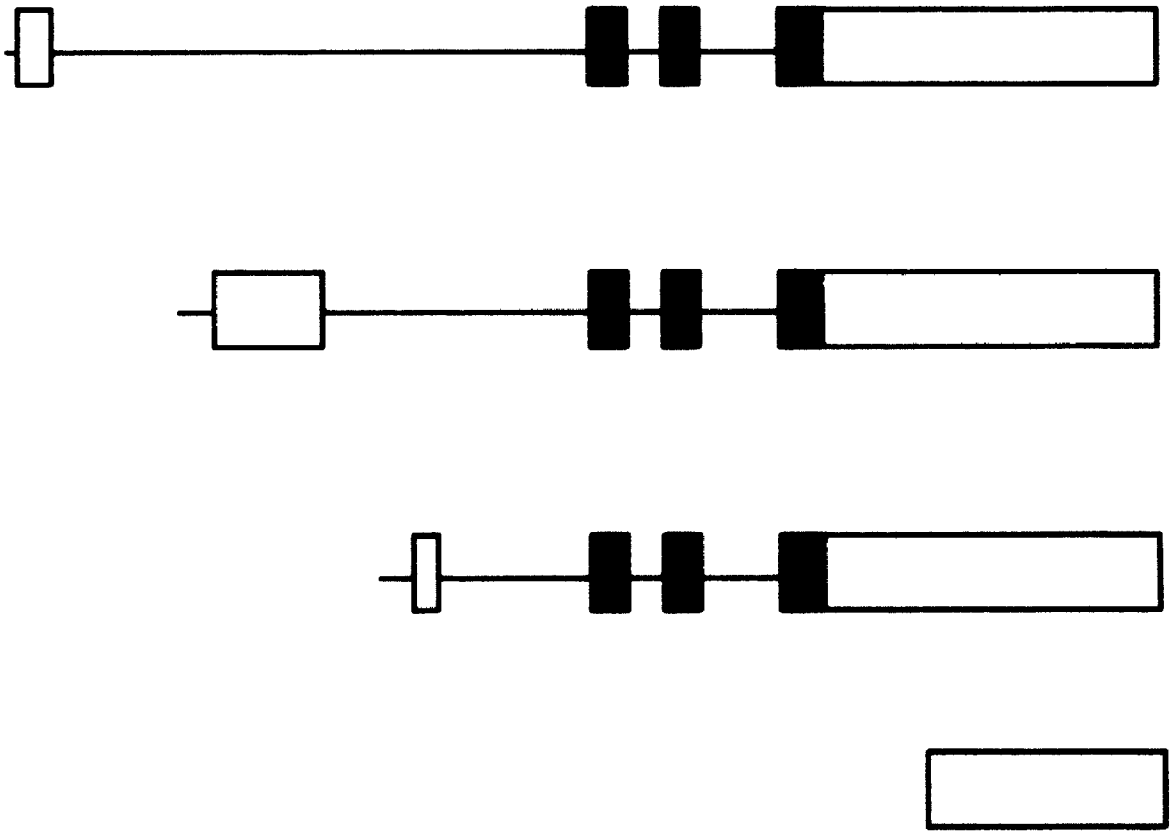
Serum IGF-II levels are high in the fetal rat and decrease after birth (Moses et al. 1980). IGF-II mRNA levels are similarly high in many fetal and neonatal tissues and decline during development except in the brain and spinal cord where tissue levels remain high (Soares et al. 1985, 1986, Brown et al. 1986, Frunzio et al. 1986, Lund et al. 1986, Stylanopoulou et al. 1988). IGF-II is normally expressed from the paternal allele while the maternal allele is silent, with the exception of the choroid plexus and leptomeninges, where

Figure 1.3 The organization of the rat IGF-II gene (**A**) and the exonic composition of the alternative IGF-II mRNAs that are generated (**B**). Exons are represented by boxes, solid lines represent introns, major polyadenylation sites are marked by asterisks, and regions encoding pre-pro-IGF-II are shaded (adapted from Ward and Elliss 1992).

A IGF-II gene structure



B The exonic compostion of IGF-II mRNAs



both alleles are expressed (DeChiara et al. 1990, 1991). In the neonatal rat, serum levels of IGF-II are reduced by fasting (Phillips et al. 1989). Glucocorticoids suppress liver IGF-II expression in the rat (Beck et al. 1988, Levinovitz and Nortstedt 1989).

1.1.3.4 Biological actions

Infusion of IGF-II in adult rats results in a weak growth promoting effect compared to the effects of IGF-I (Schoenle et al. 1985) but does induce hypoglycemia and enhances glucose uptake, glycogen formation in skeletal muscle and lipogenesis in epididymal fat (Stumpel and Hartmann 1992). IGF-II is overexpressed in many types of tumours, and large tumours which secrete IGF-II can induce hypoglycemia (Fu et al. 1988, Reeve et al. 1985, Daughaday 1990, Daughaday et al. 1988, Axelrod and Ron 1988, Christofori et al. 1994).

IGF-II overexpressing transgenic mice have variable increases in organ weight dependent upon the promoter used. Transgenic mice overexpressing IGF-II from the onset of puberty, under the control of the major urinary protein promoter, have decreased body weight due to a reduction in body fat and an increased incidence of tumours in later life (Rogler et al. 1994). IGF-II transgenic mice under the control of the phosphoenolpyruvate carboxykinase promoter did not have significant changes in body growth but exhibited increases in kidney, testis, and adrenal weight by 12 weeks of age (Wolf et al. 1994). Overexpression of an IGF-II transgene in skin, alimentary canal and uterus resulted in increased wet weight and cell number in these tissues (Ward et al. 1994).

Disruption of the IGF-II alleles in mice by gene targeting provided the first genetic evidence of a physiological role for IGF-II in embryonic growth

(DeChiara et al. 1990), and demonstrated that the IGF-II gene was imprinted (DeChiara et al. 1991). IGF-II mRNA can be detected in the mouse embryo as early as the two cell stage and has been shown to stimulate the growth and metabolism of preimplantation mouse embryos *in vitro* (Rappolee and Werb 1991, Schultz et al. 1993, Harvey and Kaye 1992, Kaye et al. 1992).

In vitro studies have demonstrated that IGF-II promotes the differentiation of variety of cell types. IGF-II stimulates the differentiation of myoblasts (Florini et al. 1991b), and promotes neurite outgrowth in cultured sensory, sympathetic, cortical and motor neurons (Recio-Pinto et al. 1984, 1986, 1988, Aizenman and deVellis 1987, Caroni and Grandes 1990),

1.2 INSULIN-LIKE GROWTH FACTOR RECEPTORS

1.2.1 Discovery

Following the purification of IGFs, it was demonstrated that they competed for binding to the insulin receptor (Hintz et al. 1972). A receptor for IGF-I was demonstrated by the specific binding of iodinated IGF-I to a receptor distinct from the insulin receptor (Marshall et al. 1974, Megyesi et al. 1975). Differences in the binding affinity of each ligand suggested that at least two receptors existed. Further binding studies demonstrated the existence of a third receptor with preferential binding affinity for IGF-II over IGF-I, and which did not bind insulin (Rechler et al. 1980). Affinity cross-linking techniques enabled the characterization of the molecular weights for the insulin, IGF-I and IGF-II receptors further adding proof to their separate identities (Massague and Czech 1982).

1.2.2 The IGF-I receptor

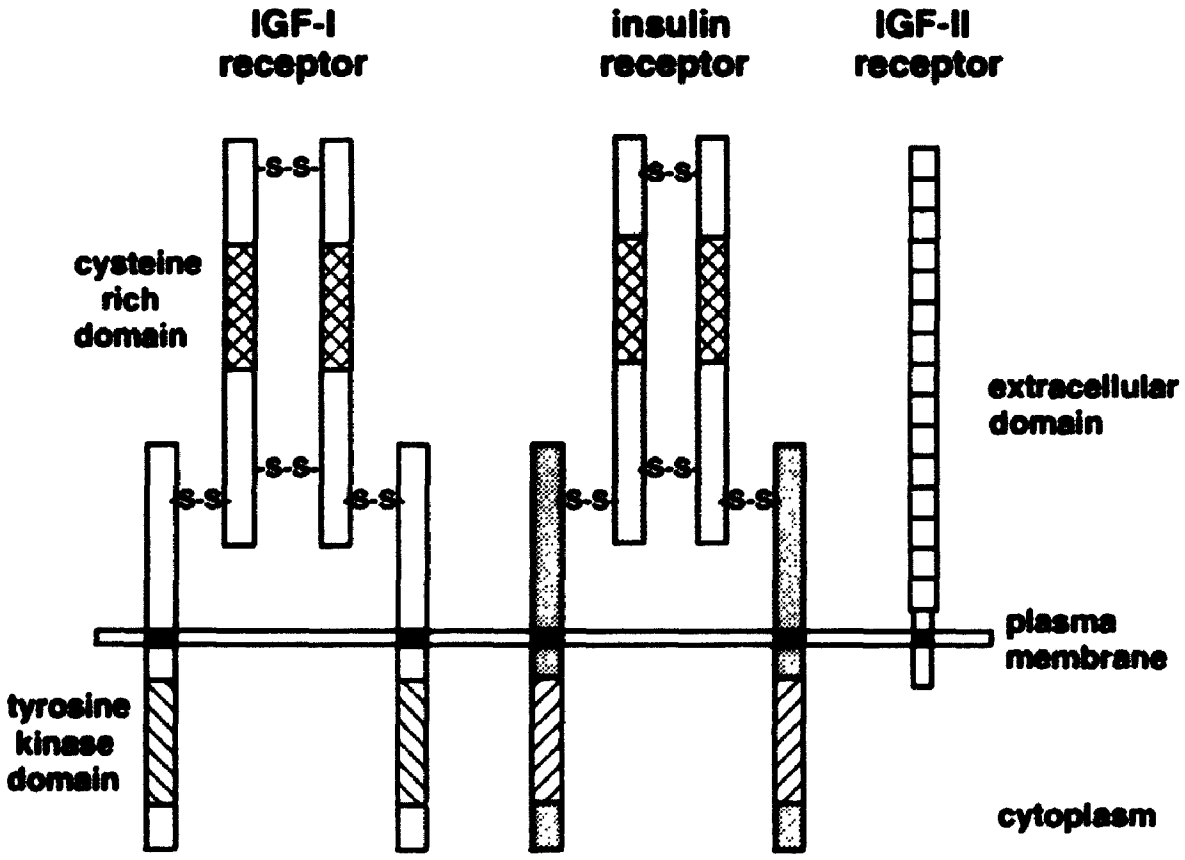
1.2.2.1 Structure

The IGF-I, or type 1, receptor (IGF1R) has a structure homologous to the insulin receptor (Chernausek et al. 1981, Kasuga et al. 1981, Massague and Czech 1982). Affinity cross-linking techniques demonstrated that it is comprised of a disulphide linked tetramer composed of two 135 000 Mr alpha-subunits and two 95 000 Mr beta-subunits. Each α- and β-subunits are linked by primary disulfide bridges and are joined to a second α- and β-chain by secondary disulfide bridges. Binding competition studies demonstrated that the IGF-I receptor binds IGF-I with highest affinity followed by IGF-II, and then insulin with 50-100 fold less affinity (Hintz et al. 1972, Marshall et al. 1974, Megyesi et al. 1975, Kasuga et al. 1981, Massague and Czech 1982) (Figure 1.4).

1.2.2.2 Interactions of IGFs with the IGF-I receptor

Domain swapping experiments and studies with antibodies that block IGF-I binding, have identified the cysteine-rich domain of the α-subunit as an important domain for high affinity IGF-I binding (Schumacher et al. 1991, Kjeldsen et al. 1991, Soos et al. 1992). Regions of the IGF molecule important for interaction with the IGF-I receptor have been identified by mutational analysis of IGF-I. The mutation of the B-chain tyrosine 24 with serine or leucine results in a dramatic reduction in the affinity of IGF-I for the IGF-I receptor (Cascieri et al. 1988a). Similarly, replacement of the corresponding B27 tyrosine in IGF-II leads to a reduction of IGF-II affinity for the IGF-I receptor. The C-domain is critical for IGF-I receptor interaction as mutations

Figure 1.4 A schematic representation of the structures of the IGF-I, IGF-II and insulin receptors. The insulin and IGF-I receptors are both heterotetrameric complexes of ligand binding α-subunits and β-subunits that contain intrinsic tyrosine kinase activity. The IGF-II receptor is structurally unrelated to the insulin and IGF-I receptors. It contains a 15 repeat extracellular domain and a short cytoplasmic domain with no tysrosine kinase activity. Shaded boxes represent the transmembrane domains, the cross-hatched regions represent the cysteine-rich regions, the hatched regions represent the tyrosine kinase domains, and -S-S- represents disulfide linkages (adapted from LeRoith et al. 1995).


IGF-I receptor
insulin receptor
IGF-II receptor
cysteine rich domain
S-S
extracellular domain
plasma membrane
tyrosine kinase domain
cytoplasm

in this domain result in up to 30-fold reduction in IGF-I receptor affinity (Bayne et al. 1988).

1.2.2.3 Gene structure

Molecular cloning of the IGF-I receptor cDNA and gene confirmed much of the biochemical characterization (Pedrini et al. 1994). The IGF-I receptor shares 50-60% overall amino acid sequence homology with the insulin receptor, and has 84% homology in the tyrosine kinase domains. The human IGF-I receptor is the product of a single gene which spans more than 100 kb, contains 21 exons, and encodes for mRNAs of 11 and 7 kb (Abbott et al. 1992). The organization of the IGF-I receptor gene reflects functional domains of the protein (Ullrich et al. 1986). Exons 1-3 code for the 5' untranslated region, the signal peptide, the non-cysteine rich N-terminal region, and the cysteine-rich domain of the α-subunit. Exons 4-10 encode for the remainder of the α-subunit. Exon 11 encodes the Arg-Lys-Arg-Arg peptide cleavage site that generate the α- and β-subunits from the polypeptide precursor. Exons 12-20 encode for the β-subunit, with the most highly conserved region between the IGF-I and insulin receptors, the tyrosine kinase domain, being encoded by exons 16-20. Exon 21 encodes for 3' untranslated region. The IGF-I receptor is expressed as 1367 amino acid precursor, which upon proteolytic cleavage, generates separate α- and β-subunits that are subsequently linked by disulfide bonds. The rat IGF-I receptor cDNA shares 98% homology with the human cDNA in the tyrosine kinase domain and 85% in the C-terminal domain (Pedrini et al. 1994).

The rat IGF-I receptor gene promoter has been cloned and found to be highly homologous to the human IGF-I receptor promoter (Werner et al. 1990a, Mamula et al. 1992, Cooke et al. 1991). It lacks TATA and CAAT boxes,

but is very GC-rich, characteristic of many "housekeeping" genes. In contrast to the insulin receptor, transcription of the rat IGF-I receptor gene is initiated at a single site 940 bp upstream of the ATG translation start site (Werner et al. 1990a). Potential binding sites for the transcription factors Sp1, ETF, GCF, and AP-2 were identified within the proximal 5' flanking region of the promoter (Werner et al. 1990a), but only Sp1 has been shown to trans-activate the rat IGF-I receptor promoter (Werner et al. 1992). Wilms Tumour-1 (WT1), a tumour suppresser gene product which generally suppresses the activity of promoters containing binding sites, has been shown to suppress the activity of the IGF-I receptor promoter (Werner et al. 1993, 1994).

1.2.2.4 Regulation of expression

Expression of the rat IGF-I receptor gene is relatively high during fetal and postnatal development and declines to lower levels during adulthood (Werner et al. 1989, Alexandrides et al. 1989, Pomerance et al. 1988). Expression of the IGF-I receptor gene is increased in the lung, stomach, kidney, and heart following fasting, and in the kidney following diabetes (Lowe et al. 1989, Werner et al. 1990b). Increases in IGF concentrations cause a decrease in IGF-I receptor number *in vitro* (Rosenfeld and Hintz 1980, Rosenfeld 1982, Yamamoto et al. 1993). The growth factors, platelet derived growth factor (PDGF) and basic fibroblast growth factor (basic FGF), both increase IGF-I receptor expression (Rosenthal et al. 1991, Rubini et al. 1994).

1.2.2.5 Signal transduction

Upon ligand binding to the α-subunit, the IGF-I receptor undergoes ligand-induced autophosphorylation. Intracellular tyrosines 1131, 1135, and 1136 in the kinase domain of the β-subunit are the primary sites for

phosphorylation and result in the activation of the intrinsic tyrosine kinase activity of the IGF-I receptor (Gronberg et al. 1993, Kato et al. 1993, 1994, Li et al. 1994). The predominant substrate of the IGF-I receptor kinase is the insulin receptor substrate-1(IRS-1), another feature common with the insulin receptor (Shemer et al. 1987, Myers et al. 1993a).

Phosphorylated IRS-1 has been shown to bind to Src homology 2 (SH2) domain containing proteins involved in signaling pathways including, phosphoinositol 3' (PI3)-kinase, Grb2, Syp, and Nck (White and Kahn 1994, LeRoith et al. 1995). These proteins activate the Ras-MAP kinase signaling pathway. Activated Ras leads to the activation of a cascade of protein kinases including Raf-1, MAP kinase kinases (MEKs), MAP kinases (ERKs), and S6 kinase, which leads to the activation of nuclear transcription factors (White and Kahn 1994, LeRoith et al. 1995). While this pathway downstream of IRS-1 has not been specifically delineated for the IGF-I receptor, there is some evidence to suggest that the activation of the IGF-I receptor leads to the phosphorylation of two proteins identified as MAP kinases (Lamphere and Lienhard 1992).

1.2.2.6 Biological effects

Studies with blocking antibodies and IGF mutants with reduced affinity for the IGF-I receptor have demonstrated that IGF-I and IGF-II exert most of their biological effects through the IGF-I receptor (Mottola and Czech 1984, Conover et al. 1986, Furlanetto et al. 1987, Bayne et al. 1988, Cascieri et al. 1988a, 1988b). This has been supported by direct genetic evidence from knockout mice. Mice homologous for a null mutation in the IGF-I receptor are phenotypically indistinguishable from mice carrying null mutations for

both IGF-I and IGF-I receptor, providing genetic evidence that the biological effects of IGF-I are mediated solely through the IGF-I receptor (Liu et al. 1993).

Overexpression of the IGF-I receptor is correlated with transformation in a number of cell types (Kaleko et al. 1990, Pietrzkowski et al. 1992a, Ambrose et al. 1994), and the transforming potential of a number of tumourigenic cell lines can be reduced by inhibiting expression of the IGF-I receptor (Sell et al. 1993, Resnicoff et al. 1994a, 1994b, Coppola et al. 1994). Transformation by the viral oncogene SV40Tag is dependent upon the presence of the IGF-I receptor as fibroblasts derived from the IGF-I receptor null mouse can not be transformed by SV40Tag (Sell et al. 1993).

1.2.3 The IGF-II receptor

1.2.3.1 Structure

The IGF-II (or type 2) receptor is a monomeric glycoprotein of 260 kDa that lacks intrinsic tyrosine kinase activity. It binds IGF-II with high affinity and IGF-I with approximately 500 fold less affinity, but does not bind insulin. The cloning of the cDNA for the IGF-II receptor predicted a protein with a large extracellular domain, a single transmembrane region and a small cytoplasmic tail (Morgan et al. 1987, MacDonald et al. 1988). Sequence comparison revealed that it was identical to the cation-independent mannose-6-phosphate receptor (Lobel et al. 1987, Kiess et al. 1988). The extracellular domain comprises about 93% of the total receptor protein, is composed of 15 repeat sequences that contain a pattern of eight conserved cysteine residues, a single fibronectin type II repeat motif and has 19 N-linked glycosylation sites (Morgan et al. 1987). A soluble form of the IGF-II receptor

generated by proteolytic cleavage of the membrane bound form has been identified in serum (Kiess et al. 1987a, Causin et al. 1988, Gelato et al. 1988).

1.2.3.2 Interactions of ligands with the IGF-II receptor

Receptors localized on the cell surface can bind mannose-6-phosphate containing glycoproteins which are then endocytosed into endosomes. Binding of IGF-II to the IGF-II receptor causes internalization and degradation of IGF-II however, the rate at which the IGF-II receptor is recycled is independent of IGF-II binding (Oka et al. 1985, Oka and Czech 1986). The binding site for IGF-II is distinct from the binding site for mannose-6-phosphate ligands, and both ligands can bind simultaneously although they can each interfere with the binding of the other (Braulke et al. 1988, Kiess et al. 1990). NMR analysis of the structure of IGF-II and comparison with the binding affinities of IGF-II mutants has revealed that amino acids Arg49, Ser50, Ala54 and Leu55, in the A domain, are essential for binding to the IGF-II receptor (Terasawa et al. 1994). Mutations of residues 49-51 in the A domain of IGF-I also reduce the affinity of IGF-I to the IGF-II receptor (Cascieri et al. 1989).

1.2.3.3 Gene structure

The rat IGF-II receptor gene has not been characterized however, the rat and mouse IGF-II cDNAs have over 90% homology and therefore would be predicted to have a similar gene structure (MacDonald et al. 1988, Szebenyi and Rotwein 1994). The mouse IGF-II receptor gene, located on mouse chromosome 17, spans 93 kb, has 48 exons which encode for a predicted protein of 2482 amino acids (Szebenyi and Rotwein 1994). The extracellular domain of the receptor is encoded by exons 1-46. Each of the 15 repeat

sequences is encoded by parts of 3-5 exons. The fibronectin type II-like element is encoded by exon 39, the transmembrane region is encoded by parts of exon 46, and the cytoplasmic region is encoded by exons 46-48.

The mouse IGF-II receptor gene is imprinted and only the maternal allele is expressed (Barlow et al. 1991) except in pre-implantation embryos where both alleles are expressed (Latham et al. 1994). Mice carrying null mutations in the maternal allele for IGF-II receptor gene die around birth and have major cardiac abnormalities demonstrating that the IGF-II receptor is crucial for normal fetal growth (Lau et al. 1994, Wang et al. 1994).

1.2.3.4 Regulation of expression

The IGF-II receptor levels are high in fetal tissues and decline in late gestation or the early postnatal period for most tissues (Sklar et al. 1989, Alexandrides et al. 1989). The circulating form of the IGF-II receptor is also developmentally regulated with high levels in fetal rat serum which decline between 20 and 40 days postnatally (Kiess et al. 1987a). IGF-I, IGF-II, insulin and epidermal growth factor increase the number of IGF-II receptors on the membrane of human fibroblasts by stimulating the redistribution of the IGF-II receptor from internal membranes to the cell surface (Braulke et al. 1989).

1.2.3.5 Signal transduction

Whether the IGF-II receptor mediates the biological response of IGF-II is unclear. *In vitro* studies using blocking antibodies for the IGF-II receptor failed to inhibit the biological actions of IGF-II (Mottola and Czech 1984, Conover et al. 1986, Furlanetto et al. 1987, Kiess et al. 1987b, Adashi et al. 1990, Hartmann et al. 1992). Most of the evidence suggests that IGF-II exerts biological activity through the IGF-I receptor as the IGF-I receptor blocking

antibody αIR-3 inhibits IGF-II mediated responses (Stracke et al. 1989, Hirai et al. 1993). However, some studies have shown that binding of IGF-II to the IGF-II receptor is coupled to guanine nucleotide-binding proteins (G proteins). It has been demonstrated in phospholipid vesicles that, IGF-II couples purified rat IGF-II receptor with guanine nucleotide inhibitory protein 2 (G_{i-2}) (Nishimoto et al. 1989) and stimulates guanosine 5'-O-(3-thiotriphosphate) (GTPγS) binding and GTPase activity of G_i proteins (Murayama et al. 1990). Other *in vitro* studies have demonstrated that a 14 amino acid segment of the IGF-II receptor can activate G_{i-2} (Okamoto et al. 1990, Okamoto and Nishimoto 1991). In addition, IGF-II stimulated DNA synthesis in 3T3 cells is a result of an increased influx of Ca^{2+} which may be due to signaling through the IGF-II receptor and coupled GTP-binding proteins (Nishimoto et al. 1987, Kojima et al. 1988).

1.2.3.6 Biological effects

The IGF-II receptor is thought to function primarily in the clearance and degradation of IGF-II. Mice heterozygous for an IGF-II receptor null allele from maternal origin, and homozygotes, die at birth with a 30% increase in birth weight compared to normal littermates and heterozygotes from paternal origin (Wang et al. 1994). These mice had organ and skeletal abnormalities and missorted mannose-6-phosphate-tagged proteins. Breeding the IGF-II mutant with the IGF-II receptor mutant resulted in rescue of the IGF-II receptor phenotype indicating that it was likely caused by an excess of IGF-II. This suggested that the main function of the IGF-II receptor, in the context of the IGF system, is in the clearance and degradation of IGF-II from the serum (Wang et al. 1994).

1.3 INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS

1.3.1 Discovery and initial characterization

During the initial characterization of IGFs from serum, it was discovered that the majority of IGF activity had a relative molecular mass of greater than 60 kDa (Koumans and Daughaday 1963). Zapf et al. (1975) demonstrated that when a radiolabeled IGF-I preparation was incubated with human serum, the radiolabeled IGF-I bound specifically to two chromatographic protein fractions of 50 and 150 kDa. The 150 kDa protein fraction was observed to be GH dependent whereas the 50 kDa protein fraction varied inversely with GH status (Moses et al. 1976, Hintz et al. 1981). When radiolabeled IGF-I was injected into the rat circulation very little free IGF-I existed, the majority of the labeled IGF-I was found to first associate in the 50 kDa protein fraction, but after 20 min to 4 h, was found mainly in the 150 kDa complex (Kaufmann et al. 1977). In addition, it was demonstrated that association of IGF-I with the 150 kDa complex extended the half-life of the radiolabeled IGF-I to 3-4 h compared to 10 min in the free form (Cohen and Nissley 1976). These studies indicated the existence of specific binding proteins for IGFs and suggested that they function to extend the half-life of IGFs in the serum.

Acid extraction of endogenous IGFs from the binding proteins was undertaken to further characterize their binding properties. Treatment at acid pH resulted in the loss of the 150 kDa complex and the association of the labeled IGF-I with protein complexes in the 40-60 kDa range. It was proposed that the 150 kDa complex was comprised of an 50 kDa acid stabile binding protein which bound an IGF molecule and an additional protein which was acid labile.

1.3.2 General structure

A family of six insulin-like growth factor binding proteins (IGFBPs) have been purified, and their cDNAs cloned and sequenced (Shimasaki and Ling 1991). The IGFBPs (Figure 1.5) are a family of structurally homologous proteins that bind both IGF-I and IGF-II with high affinity, but do not bind insulin (Jones and Clemmons 1995, Shimasaki and Ling 1991). The IGFBPs have no sequence homology with the IGF receptors. Sequence alignment of the IGFBP family members revealed regions of homology within the amino- and carboxyl- terminal thirds of the proteins (Shimasaki and Ling 1991). Except for IGFBP-6, there are 18 cysteines whose exact positions are conserved throughout this gene family. The cysteines participate in disulfide bridges which contribute to their three-dimensional structure.

The binding site(s) for the IGFs have not yet been delineated. The IGFBP gene family shares high sequence homology in their amino- and carboxyl-terminal thirds suggesting that these regions may be important for IGF binding (Shimasaki and Ling 1992). The 18 conserved cysteines are contained within these regions. Integrity of the disulfide bridges is important for IGF-I binding since reduction of IGFBPs or mutation of the conserved cysteines results in loss of IGF binding (Brinkman et al. 1991, Coulter et al. 1995). Fragments of both the amino and carboxyl-terminal regions which retain IGF binding have been described (Huhtala et al. 19486, Wang et al. 1988). Analysis of C-terminal deletion mutants of IGFBP-1 demonstrated that the C-terminal 20 amino acids was essential for IGF binding (Brinkman et al. 1991). This region contained conserved Cys-226, which upon mutation resulted in loss of IGF binding and dimer formation suggesting that the disulfide bridge in which it participated was essential for IGF binding. Systematic mutation of the conserved cysteines in the C-terminus of IGFBP-2

Figure 1.5 A schematic representation of the primary amino acid sequence of the IGFBP family. The primary sequence is divided into three regions, a highly conserved amino-terminal region, the divergent middle third region, and a highly conserved carboxyl-terminal region. The conserved cysteines are indicated by bold lines, N-linked glycosylation sites are indicated by triangles and the RGD integrin recognition motifs are indicated by the hatched region (adapted from Shimasaki and Ling 1991.


IGFBP-1
IGFBP-2
IGFBP-3
IGFBP-4
IGFBP-5
IGFBP-6

revealed that Cys-220 and Cys-232 were crucial for IGF-I binding (Coulter et al. 1995).

Regions of the IGF molecule involved in binding to the IGFBPs have been identified by mutational analysis of IGFs. Replacement of the 16 amino terminal residues in the B-domain of IGF-I with the corresponding 17 residues of insulin, completely abolishes binding to IGFBP-3 (Baxter et al. 1992, Oh et al. 1993). Removal of the first three amino acids reduces the affinity of IGF-I for IGFBPs (Forbes et al. 1988, Carlsson-Skirut et al. 1989, Oh et al. 1993). A-domain residues 48-50 and B-domain residue 26 on IGF-II have also been identified as important structural determinants of IGFBP binding (Bach et al. 1992). Other studies comparing the iodination patterns of free IGFs and IGFBP-2 bound IGFs have determined that tyrosines 60 and 59 in the carboxyl-terminals of IGF-I and IGF-II respectively may be involved in the binding to IGFBP-2 (Moss et al. 1991).

1.3.3 General biological functions of IGFBPs

The biological importance of the IGFBPs was first acknowledged when it was observed that IGFBPs prolonged the half-life of IGFs in the circulation (Cohen et al. 1976), and that IGFBPs could inhibit the insulin-like actions of IGFs in cultured cells (Zapf et al. 1979). It is now known that IGFBPs have a wide variety of functions including, i) transporting IGFs in the circulation and extracellular spaces, ii) prolonging the half-life of IGFs and regulating their clearance, iii) providing cell and tissue specific localization of IGFs, and iv) modulating the actions of IGFs with their receptors on target tissues. *In vitro* studies have demonstrated that IGFBPs may either inhibit or potentiate the biologic actions of IGFs (Zapf et al. 1979, DeMellow and Baxter 1988, Elgin et al. 1987, Blum et al. 1989, Conover 1990, Clemmons and Gardner 1990,

Jones et al. 1993). In addition to their modulation of IGF function, direct functions for IGFBPs independent of IGFs have been proposed.

1.3.4 IGFBP-1

1.3.4.1 Protein structure

IGFBP-1 was first purified from human amniotic fluid and the first ten amino acids were sequenced which provided the first structural information of an IGFBP (Pavoa et al. 1984). Isolation of a cDNA for rat IGFBP-1 revealed a primary sequence of 247 amino acids (Murphy et al. 1990). It has a molecular weight of 32 kDa under non-reducing conditions. The carboxyl-terminal domain of IGFBP-1 contains an Arg-Gly-Asp (RGD) integrin recognition motif.

1.3.4.2 Gene structure

The rat IGFBP-1 gene is 5.2 kb long and contains four protein coding exons (Unterman et al. 1992). The promoter contains TATA and CAAT boxes and putative homeodomain, AP-1, insulin and glucocorticoid response elements (Unterman et al. 1992, Goswami et al. 1994). The rat IGFBP-1 promoter contains at least one functional glucocorticoid response element and an insulin responsive element (Goswami et al. 1994). Expression of IGFBP-1 mRNA in the adult rat is high in the liver and has also been detected in the kidney, and uterus (Murphy et al. 1990, 1991).

1.3.4.3 Regulation

Circulating levels of IGFBP-1 in adult serum are normally low but are increased in fasting, diabetes, glucocorticoids and GH deficiency (Ooi et al.

1990, Seneviratne et al. 1990, Murphy et al. 1990, 1991, Luo et al. 1990). IGFBP-1 expression is decreased by insulin (Unterman et al. 1991, Orlowski et al. 1991). IGFBP-1 has been proposed to inhibit the growth-promoting and insulin-like effects of IGFs during conditions of hypoglycemia. IGFBP-1 levels are higher in the fetal circulation than the adult (Donovan et al. 1989). The expression of IGFBP-1 mRNA is restricted to the liver, kidney and uterus in the adult rat (Murphy et al. 1991). In models of fetal growth restriction, there is a dramatic increase of circulating levels of IGFBP-1 (Straus et al. 1991, Unterman et al. 1990).

Studies *in vitro* have demonstrated that protein kinase C agonists, cyclic AMP agonists, and glucocorticoids stimulate the expression of IGFBP-1 at the transcriptional level (Unterman et al. 1991, 1992, Orlowski et al. 1990a). Insulin decreases IGFBP-1 mRNA levels at the transcriptional level in fetal liver explants and a number of hepatoma cell lines (Lewitt and Baxter 1989, Conover and Lee 1990, Powell et al. 1991, Orlowski et al. 1991, Unterman et al. 1991).

1.3.4.4 Post-translation modifications

IGFBP-1 can be modified by phosphorylation of serine residues (Jones et al. 1991, Frost and Tseng, 1991). The four different phosphorylated forms of IGFBP-1 have a greater affinity for IGF-I and have been shown to inhibit IGF action while the unphosphorylated form can stimulate IGF action.

1.3.4.5 Biological activity

Infusion of supraphysiological amounts of IGFBP-1 into the rat result in a small increase of blood glucose suggesting that when present in molar excess IGFBP-1 can inhibit the glucose-lowering actions of IGF-I (Lewitt et al.

1991). In contrast, transgenic mice overexpressing IGFBP-1 under control of the mouse metallothionein promoter did not differ from normal mice in fasting or random serum glucose levels (Dai et al. 1994). These transgenic mice expressed the IGFBP-1 transgene in the brain and showed brain growth retardation from the second week of postnatal life suggesting that IGFBP-1 may inhibit IGF-I growth promoting effects during brain development at this time (D'Ercole et al. 1994). Other IGFBP-1 transgenic mouse lines have been generated with IGFBP-1 under the control of the mouse phosphoglycerate kinase promoter (Rajkumar et al. 1995). These mice showed a significant reduction in birth weight, demonstrated fasting hyperglycemia, and the brain to body weight ratio was significantly reduced compared to normal littermates. The differences observed in birth weight and fasting glucose levels compared to the other IGFBP-1 transgenic lines may be attributed to difference in tissue expression of the transgene.

In vitro studies have demonstrated that IGFBP-1 can both inhibit and potentiate IGF action. When IGFBP-1 is present in molar excess of IGF-I or IGF-II concentrations, its predominant effect is inhibition of IGF action (Busby et al. 1988, Ritvos et al. 1988, Frauman et al. 1989, Burch et al. 1990, Liu et al. 1991). IGFBP-1 potentiation of IGF stimulated DNA synthesis has been observed in smooth muscle cells, human, chicken and mouse embryo fibroblasts but only in the presence of platelet poor plasma (PPP) or low serum (Elgin et al. 1987, Clemmons and Gardner 1990). Further studies have indicated that IGF-I binding to IGFBP-1 and the IGF-I receptor is required for potentiation (Clemmons et al. 1990, Koistenen et al. 1990, Kratz et al. 1992).

Infusion of IGFBP-1 into isolated rat heart preparations has demonstrated that IGFBP-1 is capable of crossing the endothelium and this effect is enhanced if insulin is co-perfused (Bar et al. 1990). IGFBP-1 contains a

RGD integrin recognition motif which binds specifically to the $\alpha_5\beta_1$ integrin receptor (Jones et al. 1993b). Binding of IGFBP-1 to the $\alpha_5\beta_1$ integrin receptor has been correlated with the stimulation of cell migration independent of IGF-I.

1.3.5 IGFBP-2

1.3.5.1 Protein structure

IGFBP-2 was first purified from a Buffalo rat liver (BRL)-3A cell line (Lyons and Smith 1986, Mottola et al. 1986). Its primary structure was determined following isolation of its cDNA (Brown et al. 1989, Margot et al. 1989). It is a non-glycosylated protein of 270 amino acids with an apparent molecular weight of 31.5-33 kDa under non-reducing conditions. IGFBP-2, like IGFBP-1, has an RGD integrin recognition motif in its carboxyl terminal domain.

1.3.5.2 Gene structure

The rat IGFBP-2 gene consists of 4 protein-coding exons which span about 36 kb (Brown and Rechler 1990, Kutoh et al. 1993). The size of intron 1 is approximately 32 kb which accounts for the large size of this gene. The IGFBP-2 promoter lacks TATA and CAAT elements but contains three GC-rich regions. The three GC boxes are required for efficient transcription of the IGFBP-2 gene. The transcription factor Sp1 can bind to the GC boxes and directs the transcription of the IGFBP-2 gene (Kutoh et al. 1993, Boisclair et al. 1993). Other consensus sequences identified include sites for the transcription factors AP-1, AP-2 and liver factor B1.

1.3.5.3 Regulation

IGFBP-2 is the predominant binding protein found in fetal and neonatal sera, and its levels decline with increasing gestational age (Donovan et al. 1989). In addition, IGFBP-2 is expressed by many fetal tissues and declines postnatally except in the brain where expression remains at a high level in the adult. Serum IGFBP-2 is increased in IGF-I overexpressing transgenic mice and mice deficient in the IGF-II receptor gene (Camocho-Hubner et al. 1991, Lau et al. 1994). Hepatic IGFBP-2 mRNA is increased following fasting, hypophysectomy, and diabetes and is decreased by glucocorticoids (Orlowski et al. 1990, Ooi et al. 1990, 1992). Estrogen upregulates IGFBP-2 mRNA expression in the pituitary (Michels et al. 1993).

In vitro studies have demonstrated that IGFBP-2 is decreased by insulin in hepatocytes confirming *in vivo* studies where insulin infusion in the diabetic rat reduces liver IGFBP-2 levels to normal (Boni-Schnetzler et al. 1990). In a bovine kidney epithelial cell line, forskolin decreased IGFBP-2 mRNA and protein (Cohick et al. 1991). In sheep thyroid cells IGFBP-2 is increased by tetradecanoyl phorbol acetate (TPA) (Eggo et al. 1991).

1.3.5.4 Post-translational modifications

IGFBP-2 is not glycosylated as treatment with N-glycanase does not reduce its size (Zapf et al. 1988). IGFBP-2 in plasma can be cleaved by proteases that are induced by starvation (McCusker et al. 1991a).

1.3.5.5 Biological activity

IGFBP-2 has been shown to both inhibit and potentiate IGF biologic activity *in vitro*. IGFBP-2 can inhibit IGF stimulated [^{3}H]thymidine incorporation in a number of cell types. (Knauer and Smith 1980, Mottola et

al. 1986, Han et al. 1988b, Reeve et al. 1993). IGFBP-2 has been shown to be a weak potentiator. In microvascular endothelial cells, IGFBP-2 enhances IGF-I stimulation of glucose transport and amino acid uptake (Bar et al. 1989). IGFBP-2 can enhance IGF-I induced increase in DNA synthesis in porcine smooth muscle cells, but this effect is dependent upon the presence of platelet poor plasma (Bourner et al. 1992). IGFBP-2 potentiates IGF-II mitogenic activity on sheep choroid plexus cells when it is associated with the cell surface through its RGD domain (Delhanty and Han 1993). Infusion of IGFBP-2 into isolated rat heart preparations has demonstrated that IGFBP-2 is capable of crossing the endothelium (Bar et al. 1990).

1.3.6 IGFBP-3

1.3.6.1 Protein structure

IGFBP-3 is the predominant IGFBP found in adult serum and combines with an 80 kDa acid-labile subunit (ALS) and IGF-I to form the 150 kDa complex. IGFBP-3 was first isolated from human serum by Martin and Baxter (1986) and later from adult rat serum (Baxter and Martin 1987). Rat IGFBP-3 has an apparent molecular weight of 56 and 50 kDa under non-reducing conditions. Characterization of cDNAs for rat IGFBP-3 revealed a predicted 265 amino acid protein with 4 N-linked glycosylation sites (Albiston et al. 1990, Shimasaki et al. 1989). IGFBP-3 binds IGF-I and IGF-II with almost equal affinity (Baxter and Martin 1986, Martin and Baxter 1986).

1.3.6.2 Gene structure

The rat IGFBP-3 gene is a single copy gene, spans approximately 10 kb, and contains 5 exons which encode for a 2.5 kb mRNA (Albiston et al. 1995).

IGFBP-3 mRNA has been detected in adult rat liver, kidney, stomach, heart, adrenal, ovary, testis, spleen, lung and intestine (Albiston et al. 1990, Shimasaki et al. 1989). The promoter has been characterized and contains a TATA box, a GC element, and several putative response elements. Identified in the promoter region were consensus sequences for AP-2, thyroid specific transcription factor-1 and -2, ATF, nuclear factor-1, estrogen response elements, glucocorticoid response elements, and growth hormone response elements (Albiston et al. 1995).

1.3.6.3 Regulation

IGFBP-3 is positively regulated by growth hormone and IGF-I (Baxter and Martin 1989a). Its levels are low during fetal and neonatal life but increase with gestational age (Donovan et al. 1989). Serum IGFBP-3 levels are decreased by fasting, protein restriction and in diabetes (Donovan et al. 1991, Clemmons et al. 1989, Zapf et al. 1989) and are increased by dexamethasone (Luo and Murphy 1990). IGFBP-3 gene expression in the rat uterus is decreased by estradiol and tamoxifen, and increased by oophorectomy (Huynh and Pollack 1994).

Numerous *in vitro* studies have demonstrated that rat IGFBP-3 is positively induced by IGF-I in fibroblasts, mammary epithelial and sertoli cells (Conover 1990, Smith et al. 1990, Martin and Baxter 1990, Bale and Conover 1992, Romagnolo et al. 1994), by EGF in Swiss 3T3 cells and granulosa cells (Corps and Brown 1991, Mondschein et al. 1990), by TGF-β in fibroblasts (Yateman et al. 1993, Martin and Baxter 1991), by forskolin in sheep thyroid cells (Bachrach et al. 1991) and by TPA in epithelial cells (Cohick and Clemmons, 1991). Recently, it was demonstrated that the tumour suppresser protein p53 induces the expression of IGFBP-3 (Buckbinder et al. 1995).

1.3.6.4 Post-translational modifications

Rat IGFBP-3 has four N-linked glycosylation sites (Albiston et al. 1990, Shimasaki et al. 1989). The presence of glycosylation has been confirmed by the reduction of the molecular weight of IGFBP-3 upon glycanase treatment (Zapf et al. 1988). Studies in fibroblasts have determined that the glycosylation of IGFBP-3 is not required for binding to the cell surface and potentiation of IGF-I effects (Conover 1991a).

A specific IGFBP-3 protease has been described in pregnant serum, and catabolic disease states (Davenport et al. 1990, 1992a, 1992b, Cwyfan Hughes et al. 1992). Proteolytic cleavage results in the generation of a 30 kDa fragment that has reduced affinity for IGF-I (Gargosky et al. 1992). Preliminary characterization of the IGFBP-3 protease in pregnant rat serum suggests that is a calcium dependent serine protease (Davenport et al. 1990, 1992). More recent studies suggest that matrix metalloproteinases are involved in the degradation of IGFBP-3 in pregnant rat serum (Fowlkes et al. 1994).

IGFBP-3 secreted by fibroblasts and CHO cells can be phosphorylated at a two major serine residues (Hoeck and Mukku 1994). IGF-I stimulates the phosphorylation of IGFBP-3 in fibroblasts (Coverley and Baxter, 1995).

1.3.6.5 Biological activity

The principle *in vivo* function of IGFBP-3 is to transport IGF-I in the adult circulation by forming a complex with IGF-I and the ALS (Baxter and Martin 1989a, 1989b). IGFs bound in this complex are retained in the vascular circulation and have prolonged half-lives (Guler et al. 1989, Zapf et al. 1995). Infusion of an IGFBP-3-IGF-I complex lowers blood glucose and stimulates glycogen synthesis in the diaphragm of hypophysectomized rats but not normal rats (Zapf et al. 1995). In the hypophysectomized rats which lack the

150 kDa complex, the injected IGF-IGFBP-3 was found in the 40 kDa complex and was rapidly cleared suggesting that IGF associated with the 40 kDa complex can readily leave the circulation (Zapf et al. 1995).

IGFBP-3 has been demonstrated to potentiate IGF-I action *in vivo*. Subcutaneous injection of IGFBP-3 and IGF-I compared to IGF-I alone, to GH deficient rats stimulated a 2-fold greater increase in weight gain and greater epiphyseal width (Clark et al. 1993). Combined application of IGFBP-3 and IGF-I results in the acceleration of wound healing (Sommer et al. 1991, Hamon et al. 1993).

Several *in vitro* studies have shown that IGFBP-3 can inhibit IGF actions. When present in molar excess, IGFBP-3 inhibits IGF-I stimulated DNA synthesis in human fibroblasts (DeMellow and Baxter 1988), chick embryo fibroblasts (Blat et al. 1989), rat granulosa cells (Bicsak et al. 1990), and osteoblasts (Schmid et al. 1991). IGFBP-3 inhibits IGF-I stimulated glucose incorporation in BALB/c 3T3 cells (Okajima et al. 1993) and glycogenolysis and glucose oxidation in porcine fat cells (Walton et al. 1989). IGFBP-3 in solution has a higher affinity for IGFs than the does the IGF-I receptor, in contrast to its lowered affinity for IGFs when associated with the cell surface suggesting a mechanism for the inhibitory actions of IGFBP-3 (McCusker et al. 1990, 1991b).

Numerous studies have determined that IGFBP-3 can potentiate IGF actions. Pre-incubation of cells with IGFBP-3 results in the potentiation of IGF stimulated DNA synthesis in human fibroblasts (DeMellow and Baxter 1988) and baby hamster kidney cells (Baxum et al. 1989), and AIB uptake in fibroblasts (Conover 1990a, b). IGFBP-3 associates with the cell surface when pre-incubated and has a 10-fold lower affinity for IGF-I than IGFBP-3 in solution suggesting a mechanism for the potentiation of IGF actions

(Conover 1991). It was subsequently demonstrated that pre-incubation with IGFBP-3 prevented the IGF-I induced down regulation of IGF-I receptors (Conover and Powell 1991, Conover 1991b).

IGF independent actions of IGFBP-3 have been described in a number of different systems. Overexpression of IGFBP-3 in fibroblasts derived from wild type and IGF-I receptor deficient mice reduce their growth rates suggesting that IGFBP-3 reduced cellular growth independent of IGF (Cohen et al. 1993, Valentinis et al. 1995). Oh et al. (1993a) have demonstrated that exogenously added IGFBP-3 can inhibit the growth of a human breast cancer cell line independent of exogenous IGF stimulation. This inhibition was shown to be a result of IGFBP-3 interaction with a specific cell surface protein (Oh et al. 1993b). IGFBP-3 has been shown to inhibit DNA synthesis in mouse embryo fibroblasts stimulated by either 1% serum or fibroblast growth factor (Villaudy et al. 1991), and chick embryo fibroblasts stimulated by TGF-β (Imbenotte et al. 1992) or serum (Liu et al. 1992) suggesting that IGFBP-3 may inhibit the growth stimulating actions of other growth factors.

1.3.7 Acid labile subunit

The ALS was first isolated from human serum (Baxter et al. 1989) and later from rat serum (Baxter and Dai 1994). The rat ALS is a glycosylated protein of 84-86 kDa and has a sequence unrelated to IGFBPs or IGF receptors (Baxter and Dai 1994). About 80% of rat ALS contains 19-20 leucine-rich repeating units of 24 amino acids (Dai and Baxter 1992). These repeats have a consensus sequence that are characteristic of the leucine-rich protein superfamily, a group of proteins that participate in protein-protein or protein-membrane interactions (McFarland et al. 1989). Messenger RNA for ALS is most predominant in the adult rat liver (Dai and Baxter 1994).

The ALS subunit does not itself bind IGF-I but combines with an IGF-IGFBP-3 complex. The levels of circulating ALS are at least twice that of IGFBP-3 and this is thought to maintain most of the circulating IGFs and IGFBP-3 in the high molecular weight complex. This ternary complex does not cross the capillary cell barrier. How ALS is dissociated from the IGF-IGFBP-3 complex to allow for biological activity is unknown although it has been demonstrated that glycosaminoglycans can inhibit the binding of ALS with the IGF-IGFBP-3 complex (Baxter 1990). This might provide a mechanism for release of the IGF-IGFBP-3 complex at cell surface proteoglycans. ALS, like IGFBP-3, is GH dependent and circulating levels increase with gestational age (Baxter and Dai 1994). Fasting and diabetes decrease circulating ALS levels (Dai and Baxter 1994).

1.3.8 IGFBP-4

1.3.8.1 Protein structure

IGFBP-4 was first purified from adult rat serum (Shimonaka et al. 1989) and a human osteosarcoma cell line (Mohan et al. 1989). Isolation of a cDNA from rat predicted a 233 amino acid protein with one potential N-linked glycosylation site (Shimasaki et al. 1990). Its molecular weight under non-reducing conditions is 24-25 kDa for the unglycosylated form and 28-30 kDa for the glycosylated form. IGFBP-4 has two additional cysteine residues in its middle third region in addition to the conserved 18 cysteines.

1.3.8.2 Gene structure

The rat IGFBP-4 gene contains 4 exons which span at least 12 kb of the genome (Shimasaki et al. 1990, Gao et al 1993, Lin et al. 1993). The promoter

contains the conventional TATA and CAAT sequences and multiple potential cis-elements. There are three cAMP responsive elements, three AP-1 binding sites and one progesterone response element (Gao et al. 1993). A 2.6 kb IGFBP-4 mRNA transcript is expressed abundantly in the adult rat liver and lower levels are observed in the adrenal, testis, spleen, heart, lung, kidney, stomach, hypothalamus and brain cortex (Shimasaki et al. 1990).

1.3.8.3 Regulation

Serum IGFBP-4 levels are increased in IGF-II transgenic mice and in mice lacking an IGF-II receptor (Wolf et al. 1994, Lau et al. 1994). Liver IGFBP-4 mRNA expression is decreased by hypophysectomy and is partially restored by treatment with GH or IGF-I (Gosteli-Peter et al. 1994). IGFBP-4 mRNA expression in liver, heart, and skeletal and smooth muscle is decreased in diabetes (Chen and Arnqvist 1994).

IGFBP-4 gene expression is stimulated by forskolin, parathyroid hormone and parathyroid hormone-related peptide in rat osteoblast-like cells and a human osteosarcoma cell line through a cAMP dependent mechanism (Torring et al. 1991, Mohan et al. 1989). In human fibroblasts, IGFBP-4 was decreased by IGF-I and IGF-II in contrast to an epidermal squamous carcinoma cell line where IGFBP-4 was stimulated by IGFs (Neely and Rosenfeld 1992). In the rat neuroblastoma cell line B104, IGF-I increases IGFBP-4 in contrast to IGF-II which decreases IGFBP-4 (Ceda et al. 1991).

1.3.8.4 Post-translational modifications

Rat IGFBP-4 has one potential N-linked glycosylation site (Shimasaki et al. 1990). The glycosylation of IGFBP-4 has been confirmed by treatment of

IGFBP-4 with N-glycanase which reduces its molecular weight (Cheung et al. 1991). The biological significance of the glycosylation is unknown.

An IGFBP-4 specific protease that is enhanced by IGFs or dexamethasone has been described in human dermal fibroblasts, human decidual cells, neuroblastoma cells (Fowlkes and Freemark, 1992, Myers et al. 1993, Cheung et al 1994, Chernausek et al. 1995). Inhibitors of metallo-serine proteases and phorbol ester tumour promoters inhibit the IGFBP-4 protease activity (Myers et al. 1993, Cheung et al 1994, Conover et al. 1993).

1.3.8.5 Biological activity

IGFBP-4 inhibits IGF action under most experimental conditions. IGFBP-4 was isolated from a osteosarcoma cell line (Mohan et al. 1989) and a colon cancer cell line (Culouscou et al. 1991) based on its potent growth inhibiting effects. Inhibition was determined to be dose-dependent and could be overcome by increasing IGF concentrations. Additionally, IGFBP-4 was isolated from ovarian granulosa cells as a potent inhibitor of steroidogenesis both *in vitro* and when injected *in vivo* (Ui et al. 1989). IGFBP-4 inhibits IGF-I stimulated DNA synthesis in human osteosarcoma cells (Kiefer et al. 1992) and in rat neuroblastoma cells (Cheung et al. 1991). Furthermore, inhibition of the endogenous synthesis of IGFBP-4 in a human colon cancer cell line by transfection of an antisense IGFBP-4 cDNA, increased both basal and IGF-I stimulated cell growth (Singh et al. 1994).

1.3.9 IGFBP-5

1.3.9.1 Protein structure

IGFBP-5 was isolated from rat serum and its cDNA was cloned from a rat ovary library and a human placenta library (Shimasaki et al. 1991a). IGFBP-5 is a 252 amino acid protein with a molecular weight of 29 kDa under non-reducing conditions. IGFBP-5 is the most conserved IGFBP among species (James et al. 1993).

1.3.9.2 Gene structure

The rat IGFBP-5 gene spans at least 17 kb, contains 4 exons, and encodes for a mRNA of 6.0 kb (Zhu et al. 1993a). IGFBP-5 mRNA is abundantly expressed in adult rat kidney and has also been detected in testis, intestine, adrenal, stomach, spleen, heart, lung, brain and liver (Shimasaki et al. 1991a). The IGFBP-5 promoter contains both TATA and CAAT boxes in addition to putative binding sites for AP-1, AP-2, and progesterone receptor (Zhu et al. 1993a).

1.3.9.3 Regulation

IGFBP-5 is produced by a rat thyroid follicular cell line, FRTL-5, and its synthesis is increased by IGF-I, IGF-II and insulin (Backeljauw et al. 1993). IGFBP-5 gene expression is induced during terminal differentiation of the mouse C2 myogenic cell line (James et al. 1993) and during early myogenic differentiation of the IGF-I inducible C21 myogenic cell line (Rotwein et al. 1995). Similarly, IGFBP-5 expression is induced during IGF stimulated differentiation of L6 myoblasts into myotubes (Ewton and Florini 1995).

These studies indicate IGFBP-5 may have a specialized role in myoblast differentiation.

1.3.9.4 Post-translational modifications

A specific protease for IGFBP-5 has been characterized from the conditioned media of human fibroblast cultures that cleaves IGFBP-5 into non-IGF-I-binding fragments of 22, 20, 17 kDa (Nam et al. 1994). Characterization of the protease suggests that it is a calcium-dependent serine protease (Nam et al. 1994). Glycosaminoglycans have been shown to inhibit the degradation of IGFBP-5 by this protease (Arai et al. 1994). IGFBP-5 specific proteases have also been characterized in the conditioned medium of murine osteoblasts (Thrailkill et al. 1995). Proteases of 52-72 kDa that have characteristics of matrix metalloproteinase-1 and -2, and a protease of 97 kDa that does not have characteristics of matrix metalloproteinases were identified (Thrailkill et al. 1995). Serine phosphorylation of IGFBP-5 has been reported (Jones and Clemmons 1995).

1.3.9.5 Biological activity

The amount of IGFBP-5 in rat serum is physiologically insignificant. IGFBP-5 has been shown to inhibit IGF actions in a human osteosarcoma cell line when present in molar excess (Kiefer et al. 1992) and can also inhibit IGF-I induced steroidogenesis in granulosa cells (Ling et al. 1993). IGFBP-5 is associated with the cell surface of SV40 transformed human fibroblasts and is correlated with inhibition of their growth (Reeve et al. 1995).

IGFBP-5 can associate with the extracellular matrix of fibroblast cells. Extracellular matrix associated IGFBP-5 can potentiate the growth stimulatory effects of IGF-I possibly due to its seven-fold lower affinity for IGF-I compared

to IGFBP-5 in solution (Jones et al. 1993). IGFBP-5 has also been shown to potentiate the IGF-II stimulated action of osteoblast cells (Bautista et al. 1991).

1.3.10 IGFBP-6

1.3.10.1 Protein structure

The human IGFBP-6 was first purified from human cerebrospinal fluid and was reported to have 10-fold greater affinity for IGF-II than IGF-I (Roghani et al. 1989). Rat IGFBP-6 has a molecular weight of 34 kDa under non-reducing conditions. The cDNA for rat IGFBP-6 predicted a protein of 201 amino acids with only 14 cysteines (Shimasaki et al. 1991b). The absence of these cysteines results in the loss of the consensus sequence Gly-Cys-Gly-Cys-Cys found in all of the other IGFBPs. It is thought that the loss of this sequence contributes to the preferential binding of IGF-II.

1.3.10.2 Gene structure

The rat IGFBP-6 gene contains 4 exons that span 5.1 kb of the genome (Zhu et al. 1993b). Expression of the 1.6 kb IGFBP-6 mRNA has been reported in adult rat lung, testis, intestine, adrenal, kidney, stomach, spleen, heart, brain and liver (Shimasaki et al. 1991b). The promoter does not contain TATA or CAAT boxes, is not particularly GC-rich, but has putative response elements for Sp1, an estrogen receptor, and retinoic acid (Zhu et al. 1993b).

1.3.10.3 Regulation

Very little is known about the regulation of IGFBP-6. IGF-II has been reported to stimulate the expression of IGFBP-6 in NIH 3T3 cells (Claussen et

al. 1995). IGF-I has been reported to stimulate the expression of IGFBP-6 in L6E9 myoblasts (Silverman et al. 1995).

1.3.10.4 Post-translational modifications

The IGFBP-6 is O-glycosylated as evidenced by its reduction in size by incubation with O-glycanase (Bach et al. 1993).

1.3.10.5 Biological activity

It is thought that IGFBP-6 may function to protect IGF-I producing cells from IGF-II action since it has a selective affinity for IGF-II. In support of this hypothesis, IGFBP-6 has been shown to inhibit the IGF-II induced differentiation of L6A1 myoblasts in culture (Bach et al. 1994).

1.4 THE IGF SYSTEM IN DEVELOPMENT

1.4.1 The IGF system genes are expressed during development of the rodent

Both IGF-I and IGF-II mRNAs are expressed in many tissues during development prior to the onset of GH dependence (Lund et al. 1986, Rotwein et al. 1987, Lee et al. 1990, Streck and Pintar 1992). The lack of GH induced circulating IGF-I and the tissue specific localization of IGFs suggests an autocrine/paracrine mode of action for IGFs in development . The IGF receptors are expressed fairly ubiquitously throughout development following implantation and during early postnatal life (Bondy et al. 1990, Werner et al. 1990). The family of IGFBPs are expressed during embryonic development, each with an unique, although sometimes overlapping, pattern of expression suggesting, that the IGFBPs modulate IGF action in a

tissue specific manner, and may have unique, as well as shared, roles in normal embryonic development (Tseng et al. 1989, Wood et al. 1992, Streck et al. 1992, Cerro et al. 1993, Green et al. 1994, Schuller et al. 1993).

1.4.2 Evidence from gene targeting for the role of the IGF system in development

Gene targeting experiments have elegantly demonstrated a requirement for IGF-I, IGF-II, IGF1R, and IGF2R for normal prenatal growth and development. Mice heterozygous for a mutant IGF-II paternal allele or homozygous for mutant IGF-I alleles have a 60% reduction in birth weight compared to normal littermates while maintaining relative body proportions (DeChiara et al. 1990, 1991, Liu et al. 1993). Mice carrying a mutation in both the IGF-I and IGF-II genes die within minutes of birth due to respiratory failure, with a birthweight 30% of wild type littermates (Liu et al. 1993). Another group has produced mice lacking functional IGF-I which also display growth retardation but most die at birth from respiratory failure (Powell-Braxton et al. 1993).

Mice lacking a functional IGF-I receptor gene die immediately after birth with birthweights 45% of controls, generalized organ hypoplasia and developmental delays (Liu et al. 1993). The IGF-I receptor mutant was phenotypically indistinguishable from the IGF-I/IGF-I receptor double mutant, providing genetic evidence for the sole interaction of IGF-I with the IGF-I receptor (Lui et al. 1993). The IGF-I/IGF-II double mutants were indistinguishable from IGF-II/IGF-I receptor double mutants in that both had further growth deficiencies compared to the IGF-I receptor mutant and the IGF-I/IGF-I receptor double mutant suggesting that IGF-II interacts with another receptor to transduce a growth signal (Liu et al. 1993).

Mice heterozygous for an IGF-II receptor null allele from maternal origin and homozygotes, die at birth with a 30% increase in birth weight compared to normal littermates and heterozygotes from paternal origin (Wang et al. 1994). These mice had organ and skeletal abnormalities and missorted mannose-6-phosphate-tagged proteins. Breeding the IGF-II mutant with the IGF-II receptor mutant resulted in rescue of the IGF-II receptor phenotype indicating that it was likely caused by an excess of IGF-II (Wang et al. 1994).

Mice lacking a functional gene for IGFBP-2 have no developmental delays or defects (Wood et al. 1993). This result was suprising since IGFBP-2 is considered the predominant IGFBP during fetal life and has very distinct sites of expression that correlate with developmental processes in a number of tissues (Tseng et al. 1989, Wood et al. 1992, Lee et al. 1993). These results suggest that members of the IGFBP gene family may functionally compensate for each other during development.

1.5 DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

1.5.1 Overview of the development of the rodent central nervous system

Development of the nervous system first arises by formation of the neuroepithelium, which appears as a slight depression along the dorsal midline of the embryo, in response to signals from the underlying mesoderm. The neuroepithelium encloses upon itself to form the neural tube which generates most of the nervous system. At the time of neural tube closure, the dorsal aspect gives rise to a migrating cellular population, the neural crest (Bartlett et al. 1994). The neural crest generates most of the

peripheral nervous system and a variety of other cell types including, melanocytes and adrenal medullary cells (Jessell and Schacher 1991, Arenander and DeVellis 1994).

Development of the rat cerebral cortex proceeds from the early embryonic stages through postnatal life and reaches maturity by about 4 weeks of life. The initial phase of development is characterized by proliferation of the neuroepithelium causing it to become thickened in appearance. This ventricular zone is comprised of progenitor neuronal and glial cells which are actively proliferating and at various developmental stages stop proliferating and migrate to the outer regions of the cortex. Neuronal migration is assisted by radial glia which extend processes and maintain contact with the inner ventricular zone and outer pial region of the cortex. Soon after the formation of the ventricular zone, the marginal zone, or primordial plexiform layer, is formed at the pial surface which contains the first layer of neurons to differentiate. As post-mitotic neurons migrate out from the ventricular zone to the pial surface, an intermediate zone develops between the ventricular and marginal zones. During migration, neurons will stop at the junction between the intermediate and marginal zones to form the cortical plate. At the base of the intermediate zone, a second proliferative zone develops termed the subventricular zone. With the exception of the original marginal zone neurons, the cortical plate develops within the marginal zone by splitting into superficial and deep parts. The neural layers are formed in an inside-out fashion in that the earliest born neurons are located in the deepest parts of the cortex, while later born neurons migrate past and reside in more superficial layers (McConnell 1988, Rakic 1988).

Astroglial cells are also derived from the proliferating ventricular zone. The first astroglial cells to differentiate are the radial glia which assist

in neuronal migration. Presumptive astroglia are found in the ventricular, subventricular and marginal zone stem cells (Federoff 1986). Astrocyte proliferation generally occurs at a later developmental stage than neuronal proliferation. Astrocyte differentiation in the rat occurs mainly in postnatal life and may continue up to 30 days postnatum. There are generally two types of astrocytes which differ in appearance and location. The fibrous astrocyte has a greater number of glial intermediate filaments composed of glial fibrillary acidic protein and have long processes. It is found predominantly in the white matter of the brain where there is a high density of myelinated axons. The protoplasmic astrocyte has fewer glial intermediate filaments, shorter processes and are found predominantly in the grey matter, around nerve cell bodies, dendrites, and synapses (Steward 1989).

1.5.2 Astroglial cell function

Astroglia have important functions in the development of the CNS, maintenance of homeostasis, and repair following injury (Steward 1989). During development, astroglia provide a scaffolding for the migration of neurons from the proliferative ventricular surface of the neural tube to outer regions of the CNS. They are a source of trophic factors for neurons and aid in maintaining cellular homeostasis by buffering the extracellular environment. Astroglia possess uptake mechanisms for the removal of K^+ and neurotransmitters from the extracellular environment generated by neuronal activity (Steward 1989). They provide a mechanism for exchange of cytoplasmic material between cell types due to their extensive coupling by gap junctions (Mugnaini 1986). Following injury, astroglia undergo proliferation and hypertrophy to form glial scars in spaces left vacant by dying tissue, and remove debris by phagocytosis.

1.6 THE IGF SYSTEM IN THE CENTRAL NERVOUS SYSTEM

1.6.1 Expression of IGFs in the CNS

IGF activity in brain tissue was first detected in the culture medium of fetal brain tissue explants (D'Ercole et al. 1980) and was first purified from human fetal brain tissue by Sara et al. (1986). IGF-I and IGF-II mRNAs were first detected in the developing fetal rat brain by Lund et al. (1986) confirming the local production of IGFs in the developing brain. Numerous studies have since confirmed the expression of IGF-I and IGF-II genes during pre- and post-natal development of the rat brain (Andersson et al. 1988, Rotwein et al. 1988, Wood et al. 1990, Ayer-Le Lievre et al. 1991, Bach et al. 1991, Lee et al. 1992, 1993).

1.6.1.1 Cellular localization of IGF-I

IGF-I mRNA expression has been localized to the cerebellum, olfactory bulb, thalamus, hippocampus, pituitary, and retina (Rotwein et al. 1988, Andersson et al. 1988, Bach et al. 1991, Bach and Bondy 1992, Bondy 1991, 1992). IGF-I mRNA has been localized to the Purkinje cells of the cerebellum (Bondy 1991), and retinal ganglion cells (Lee et al. 1992) during their period of peak postnatal maturation, by *in situ* hybridization. IGF-I mRNA expression was also found in specific nuclei involved in the cerebellar relay system and in synaptic stations of the developing olfactory, auditory, visual, and somatosensory systems (Bondy 1991, Ayer-Le Lievre et al. 1991). In each system, peak expression occurs during a [illegible] stage of development characterized by dendritic maturation and synapse formation suggesting that IGF-I may be involved in the formation of system-specific synapses or myelinization. IGF-I mRNA in the adult brain has been detected in the

olfactory bulb, hippocampus and cerebellum (Werther et al. 1990, Bach et al. 1991, Bondy 1991). Immunohistochemical studies have identified IGF-I immunoreactive material in areas that correlate with the sites of synthesis as determined by *in situ* hybridization (Andersson et al. 1988, Garcia-Segura et al. 1991).

1.6.1.2 Cellular localization of IGF-II

IGF-II mRNA is first detected in the rat at embryonic day 10 in the head mesenchyme (Stylianopoulou et al. 1988a, Lee et al. 1990). In addition, IGF-II mRNA is highly expressed from early development to maturity, in areas of vascular interphase with the brain such as the choroid plexus, meninges, and blood vessels (Stylianopoulou et al. 1988b, Hynes et al. 1988, Wood et al. 1990, Ayer-Le Lievre et al. 1991, Bondy et al. 1990, 1992). In addition, IGF-II mRNA was detected in the hypothalamus, the pineal primordium at embryonic day 18 and in Rathke's pouch (Stylianopoulou et al. 1988a, Ayer-Le Lievre et al. 1991). IGF-II expression remains at a high level in the choroid plexus and meninges of the adult rodent brain (Hynes et al. 1988, Stylianopoulou et al. 1988b) where it may contribute to the very high level of IGF-II found in the CSF.

Immunocytochemical studies have confirmed the presence of IGF-II peptide in the mesenchymal structures of the choroid plexus and in the leptomeninges (Sullivan and Feldman 1994). In addition, IGF-II immunoreactivity can be detected transiently in the presumptive glia of the hippocampus and medial basal hypothalamus, and in a small population of neurons in the brain stem (Sullivan and Feldman 1994).

1.6.2 The IGF receptors in the CNS

IGF-I and IGF-II receptors were first detected in brain tissue by immunocytochemical and binding studies of brain homogenates (Gammeltoft et al. 1985, Ocrant et al. 1988). Biochemical characterization determined that structural variants of the IGF-I receptor existed (Heidenreich et al. 1986, Burgess et al. 1987, Shemer et al. 1987a, Ocrant et al. 1988). The brain IGF-I receptor has a 10 kDa smaller α-subunit which has been mainly attributed to differences in glycosylation. A structural variant of the IGF-II receptor has also been identified in the rat which also has a lower molecular weight due to differences in glycosylation (McElduff et al. 1987). In culture, neuronal cells express the brain variant of the IGF-I receptor while glial cells express the nonneuronal IGF-I receptor (Han et al. 1987b, Shemer et al. 1989).

1.6.2.1 Cellular localization of IGF receptors

The IGF-I receptor is expressed in a constitutive manner throughout the brain and shows spatio-temporal increases in conjunction with IGF-I expression during specific developmental periods (Werner et al. 1989, Bondy et al. 1992). IGF-I receptor is highly expressed during postnatal development in specific sets of sensory and cerebellar projection neurons which corresponds to the expression of IGF-I in these areas (Bondy et al. 1992). IGF-I receptor mRNA expression is higher in neuron rich regions compared to glial enriched areas of the cortex and during postnatal development when the proportion of glial cells increase, expression declines (Pomerance et al. 1988, Bondy et al. 1992). In the adult rat brain, IGF-I receptor mRNA expression has been detected in the hypothalamus, olfactory bulb, hippocampus and cerebellum (Matsuo et al. 1989, Werther et al. 1990, Aguado et al. 1993).

Specific binding of IGFs in the adult rodent brain has been demonstrated *in vivo* by autoradiographic techniques (Bohannon et al. 1986, 1988, Lesniak et al. 1988, Werther et al. 1989, Araujo et al. 1989, Pons et al. 1991). Sites of IGF binding correlate well with localization of IGF receptor mRNA expression by *in situ* hybridization (Bondy et al. 1992, Marks et al. 1991).

1.6.3 IGFBPs in the CNS

IGFBPs were first identified in rat pituitary gland and brain explants by Binoux et al. (1981). Subsequently, IGFBPs were isolated and characterized from cerebrospinal fluid and neuronal and glial cell cultures (Ocrant et al. 1990, Tseng et al. 1989, Lamson et al. 1989, Rosenfeld et al. 1989, Romanus et al. 1989, Hossenlopp et al. 1986a, Roghani et al. 1989). IGFBP-2 and IGFBP-6 have been purified from human cerebrospinal fluid and both exhibit a preferential binding affinity for IGF-II (Roghani et al. 1989, 1991a, 1991b). IGFBP-2 is the predominant IGFBP in the brain and cerebrospinal fluid (Ocrant et al. 1990).

1.6.3.1 Cellular localization of IGFBPs

In early development, IGFBP-2 has been localized to the neural tube following neurulation and in mid-gestation is found in the epithelium of the choroid plexus, the floor plate, and the infundibulum (Tseng et al. 1989, Wood et al. 1992, Sullivan and Feldman 1994). IGFBP-5 is expressed in the floor plate and the neuroepithelium during early development and in the mitral cell layer of the olfactory bulb at mid-gestation (Russo et al. 1994, Green et al. 1994). At embryonic day 10, IGFBP-2 mRNA is expressed in the rostral

brain and IGFBP-5 mRNA is expressed in the midbrain and caudal neural tube (Green et al. 1994). Late in development, IGFBP-2 and IGFBP-5 are expressed synchronously with IGF-I, and have coordinate expression in neurons and glia of the olfactory bulb, cerebellar and retinal systems. IGFBP-2 tends to be expressed primarily in glial cell types while IGFBP-5 is expressed in neuronal populations (Lee et al. 1992, Bach and Bondy 1992, Lee et al. 1993, Bondy and Lee 1993). In the embryonic day 11 mouse, IGFBP-4 mRNA was detected in the telencephalon and mesencephalon (Schuller et al. 1993). In the rat, IGFBP-4 mRNA is detected in the hippocampus by embryonic day 20 and in the cortex by postnatal day 4 (Brar and Chernausek 1993).

In the adult rat, IGFBP-2 mRNA is localized to the leptomeninges, choroid plexus, parenchymal microvasculature, the epithelial cell lining of the anterior lobe and the neural lobe of the pituitary (Logan et al. 1994, Bach and Bondy 1992, Lee et al. 1993). IGFBP-4 mRNA is expressed in the hippocampus and throughout the cortex (Stenvers et al. 1994). IGFBP-5 mRNA is expressed in the principal neurons of the anterodorsal nucleus, forebrain white matter tracts, olfactory nerve, thalamic nuclei, leptomeninges and perivascular sheath (Bondy and Lee 1993, Stenvers et al. 1994).

1.6.4 The *in vivo* actions of IGF in the CNS

Transgenic mouse models have provided very strong genetic evidence for a functional role of IGF-I in myelination. Transgenic mice that overexpress IGF-I have disproportionately increased brain to body weight ratios (Mathews et al. 1988a, 1988b). By postnatal age day 55 when brain growth and myelination are essentially complete, the brains of the IGF-I transgenic line were 55% larger than normals. This increase in brain weight was attributed to an increase in cell size and number. In addition, total

myelin content was 130% greater in the transgenic compared to the controls (Carson et al. 1993). Mice homozygous for null alleles of IGF-I have reduced brain weights, 38% below age-matched wild type mice (Beck et al. 1995). There was a reduction in the size of white matter structures due to decreased numbers of axons and oligodendrocytes. In addition, the Snell dwarf mouse, which is growth hormone deficient and thus has reduced levels of IGF-I, is hypomyelinated in the CNS and has fewer oligodendroglia (Noguchi et al. 1982).

Mice carrying null mutations of the IGF1R also show developmental abnormalities in the CNS. The cellular density in the mantle zone of the spinal cord was found to be significantly higher in mutant than wild type littermates at embryonic day 14.5-18.5 (Liu et al. 1993). The authors suggested that the increase in cellular density was due to a crowding of neural cells as a result of a reduction in the amount of the surrounding neuronal fibres and neuroglia and not due to an absolute increase in cell number.

1.6.5 The *in vitro* actions of IGFs on neuronal and glial cells

Numerous investigators have demonstrated that IGFs stimulate mitogenesis in astroglial cells (Lenoir and Honneger 1986, Han et al. 1987, Shemer et al. 1987a, Ballotti et al. 1987) and neuronal cells (Shemer et al. 1987a, DiCiccio-Bloom and Black 1988, Carlsson-Skwirut et al. 1989, Nielsen et al. 1991, Torres-Aleman et al. 1988, 1989, Feldman and Randolph 1991). IGFs can also promote the survival of neurons (Aizenman and deVellis 1987, Caroni and Grandes 1990).

IGFs also have effects on the differentiation of neuronal and glial cells. IGFs have been shown to promote neurite outgrowth and stimulate the synthesis and expression of neurofilament and tubulin proteins in neuronal

cultures (Recio-Pinto et al. 1984, 1986, 1988, Feldman and Randolph 1991, Wang et al. 1992). IGF-II, and IGF-I with less potency, enhanced choline acetyltransferase activity in mouse septal neurons (Konishi et al. 1994). IGF-I induces the differentiation of oliogodendroglia from the oligodendroglial/type 2 astrocyte O2A precursor cells (McMorris et al. 1986, McMorris and Dubois-Dalq 1988). IGF-I can stimulate the uptake of 2-deoxy-glucose in primary neonatal astrocytes (Masters et al. 1991).

1.6.6 The involvement of the IGF system in the brain injury response

Injury to the CNS, whether initiated by ischemia, mechanical forces or disease, can initiate a cascade of events characterized by selective neuronal loss, wound repair by reactive astroglial cells and axon resprouting which may lead to functional recovery. Astroglia respond to injury by proliferating to fill in the wound site and are termed reactive astroglia. Reactive astroglia express a number of growth factors during injury which are thought to stimulate their own proliferation and aid in axonal regeneration. The IGF system has been shown to play an important role in the response to brain injury (Reier 1986, Lindsay 1986, Steward 1989).

Astroglial cells abundantly express IGF-I mRNA, IGF-I immunoreactivity and IGF-II immunoreactivity following ischemic injury (Gluckman et al. 1992, Garcia-Estrada et al. 1992, Beilharz et al. 1995). Yamaguchi et al. (1991) have reported an accumulation of extracellular immunoreactive IGF-I in the brain following an electrolytical lesion. In addition, astroglia cells express abundant IGF-I mRNA following cryogenic spinal cord injury (Yao et al. 1995) and demyelination induced by cuprizone (Komoly et al. 1992) and autoimmune encephalomyelitis (Liu et al. 1994). Upregulation of IGF expression in astroglial cells following injury suggests

that they may be actively involved in the processes of neuronal survival and remyelination. Evidence to support this hypothesis has been provided by intraventricular injection of IGF-I which has been demonstrated to reduce the loss of neurons following ischemic injury (Gluckman et al. 1992, Guan et al. 1993, Zhu and Auer 1994).

Injury to the brain also results in changes in expression of IGFBPs. IGFBP-2 expression is increased in the lesioned area following ischemic injury and the level of upregulation appears to correspond to the severity of the injury (Klempt et al. 1992, 1993). IGFBP-2 expression is also upregulated in astroglia following cryogenic spinal cord injury (Yao et al. 1995) and mechanical injury (Logan et al. 1995, Sandberg Nordqvist et al. 1995). IGFBP-5 expression is upregulated in the lesioned site following severe ischemic injury (Beilharz et al. 1993) and in astroglia and neurons following mechanical injury (Logan et al. 1995). IGFBP-4 expression has been reported to be reduced in the lesion site following ischemia (Beilharz et al. 1993) and slightly upregulated following cortical contusion (Sanberg Nordqvist et al. 1995). These results suggest that IGFBPs, particularly IGFBP-2 and IGFBP-5, may modulate the IGF-I induced repair following CNS injury.

1.12 SCOPE OF THESIS

1.12.1 Hypothesis

As discussed above, the IGF system is thought to have an important role in the growth and differentiation of neurons and astroglia in the development of the brain. In view of the importance of the astroglial cell to the development of the brain, this thesis has focused on the IGF system in the

growth of astroglial cells. More specifically, the role of the IGFBPs in modulating the growth promoting actions of IGFs on astroglial cells has largely been uncharacterized. We hypothesized that astroglial IGFBPs are regulated by growth factors produced locally in the brain by autocrine or paracrine mechanisms and that the IGFBPs modulate the growth promoting actions of IGFs and may have possible direct effects on growth of astroglial cells in the developing rodent brain.

1.12.2 Objectives

The regulation of the synthesis of IGFBPs would have important effects in modulating the biologic actions of IGFs on astroglia or other neighbouring cell types and thus have important consequences on the growth and differentiation of developing brain tissues. In order to determine the factors that regulate the biosynthesis of IGFBPs produced by astroglial cells, primary cultures of astroglial cells from neonatal rats were chosen as a m ·'el system. These cells synthesize IGF-I, IGF-II, both receptors, and IGFBP-2 and IGFBP-3 (Han et al. 1987, 1988, Ocrant et al. 1989, 1990, Olson et al. 1991). In addition, these cells respond mitogenically to exogenous IGFs (Enberg et al. 1985, Lenoir and Honneger 1986, Han et al. 1987, Shemer et al. 1987, Ballotti et al. 1987) and thus provide an excellent *in vitro* model for examining components of the IGF system. The **objectives** included;

i) **to determine whether the endogenous ligands IGF-I, IGF-II and insulin regulate the synthesis of IGFBPs**

ii) **to determine whether other growth factors produced locally in the brain regulate the synthesis of IGFBPs**

iii) **to determine whether intercellular communication, also known to effect the growth of astroglial cells, regulates the synthesis of IGFBPs**

IGFBP-2 is the predominant IGFBP expressed in the developing and adult brain, is synthesized by astroglial cells and can inhibit the IGF induced mitogenesis in primary astroglial cells when added exogenously (Han et al. 1988). The role of endogenously produced IGFBP-2 in modulating the growth of astroglial cells is hypothesized to be important in the overall development of the brain. The fourth objective was therefore;

iv) **to determine the role of endogenously produced IGFBP-2 in regulating the growth of astroglial cells.**

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Peptides

Human recombinant IGF-I, human recombinant IGF-II, and human recombinant TGF-α were purchased from Bachem Co. (Torrance, CA), insulin from Sigma Chemical Co. (St. Louis, MO), and human recombinant basic FGF, human recombinant acidic FGF, and human recombinant EGF from Upstate Biotechnology Incorporated (Lake Placid, NY). Human recombinant IGFBP-5 was purchased from Austral Biologicals (San Ramon, CA).

2.1.2. Antibodies

The bovine IGFBP-2 antiserum was purchased from Upstate Biotechnology Incorporated. The glial fibrillary acidic protein (GFAP) monoclonal antibody was purchased from Boehringer Manheim Canada (Laval, Que). The connexin43 antiserum was a gift from Dr. B. Nicholson, State University of New York, Buffalo, NY. An EXTRA-3 rabbit ExtrAvidin® Peroxidase Staining Kit was purchased from Sigma Chemical Co.

2.1.3 Reagents

Hexadimethrine bromide [1,5-Dimethyl-1,5-diazaundecamethylene polymethobromide (polybrene)] was purchased from Sigma Chemical Co. Fetal bovine serum (FBS), penicillin/streptomycin, gentamycin, actinomycin-D, heparin, GRGDSP peptide and Geneticin® (G418-Sulfate) were purchased from GIBCO/BRL (Grand Island Biological Co, Grand Island, NY). Poly-L-lysine was purchased from ICN (St. Laurent, Que.).

2.2 Cell Culture

2.2.1 Preparation of primary astroglial cultures

Primary rat astroglial cultures were prepared according to the methods of McCarthy and deVellis (1980). Two to three litters of 1 d-old rats (Sprague-Dawley crossed with Long Evans; Charles River Breeding Laboratories, Montreal, Quebec) were decapitated, the heads were placed in petri dishes containing ice-cold saline I (Appendix I). The brain was exposed by aseptic dissection and an approximately 6 mm x 3 mm x 3 mm wedge of tissue was dissected from each cerebral hemisphere and placed in a petri dish containing ice-cold saline I. The meninges were removed with the aid of a dissecting microscope (Olympus TL2, Carsen Medical and Scientific Co. Ltd., Markham, Ont.). The saline I was aspirated and the tissue was minced with scissors until very fine. The tissue was incubated for 20 min at RT in a solution of 0.25% (v/v) trypsin-EDTA (GIBCO/BRL) and 0.05 mg/ml DNAse I (GIBCO/BRL). Digestion was stopped by the addition of complete medium [Basal Medium Eagle (BME, GIBCO/BRL) supplemented with 10% FBS (GIBCO/BRL), penicillin (5 U/ml), streptomycin (5 µg/ml), and dextrose (6.25 g/L)], the tissue was mechanically triturated, filtered through a 130 µm Nytex monofilament screen (Tetko, Elmsford, NY), and rinsed twice with complete medium. The cells were pelleted in a table top Dynac II centrifuge (Becton, Dickinson and Co.) and resuspended in complete medium. Cell viability and counts were determined by the trypan-blue exclusion method. A cell suspension of 2 x 10^7 live cells/10 ml was plated in T-75 tissue culture flasks (75 cm^2 growth area, Costar Corporation, Cambridge, MA) and cultured in a humidified incubator (Model 1820IR, VWR Scientific, Toronto, Ont.) at 37°C in 5% CO_2 and 95% air for 10-12 d, until confluent.

Contaminating oligodendroglial cells were removed by rotary shaking. The mixed cell cultures were fed fresh complete medium, and were allowed to equilibrate for at least 1 h. The lids were tightened completely, the flasks placed in a Lab-line® orbit environ-shaker (Lab-Line Instruments, Melrose Park, Il) equilibrated to 37°C, and shaken at 2 700 rpm for 16 h. The flasks were removed from the shaker and the free floating oligodendroglial cells were removed by aspiration. The cells were rinsed with saline I and then fed with complete medium. The astroglial cells were used within two weeks of enrichment, and at least one day after the media change.

Immunofluorescence with an antibody against glial fibrillary acidic protein (GFAP) was used to characterize the primary cultures. Cells were plated at a density of 2 x 10^5 cells/ml onto 8-well Tissue-Tek chamber slides (Nunc, Roskilde, Denmark) and cultured in complete medium for 48 h. The medium was aspirated, the cells were washed with ice cold Dulbecco's phosphate buffered saline (DPBS; GIBCO/BRL) and fixed in 4% paraformaldehyde, 0.2% glutaraldehyde in phosphate buffered saline (PBS; Appendix I) for 30 min at RT. The cells were washed 3 x 10 min in PBS, solubilized with 0.05% (w/v) saponin (Sigma Chemical Co.) in Hanks Balanced Salt Solution (Sigma Chemical Co.), washed 3 x 10 min in PBS, and incubated with a mouse monoclonal antibody raised against GFAP at a dilution of 1:500 in antibody diluting solution (Appendix I) at 4°C for 16-18 h. The cells were washed 3 x 10 min in PBS and incubated with flourescein conjugated horse anti-mouse IgG (Vector Laboratories Inc., Burlingame, CA) at a 1:10 dilution in antibody diluting solution for 2 h at RT. The cells were washed 3 x 10 min in PBS, the chambers were removed, and the slides were wet mounted with Crystal/Mount (Biomeda Corp., Foster City, CA). The cultures consisted of more than 90% GFAP-immunopositive polygonal cells.

The remaining cells consisted of process-bearing cells (GFAP-positive cells and some oligodendrocytes), ependymal cells, and fibroblasts. The morphologies of the two populations of GFAP-positive astroglial cells, flat polygonal (non process bearing) and round (process bearing) are shown in Figure 2.1.

2.2.2 Maintenance of C6 cell lines

Wild type C6 glioma cells and three clones stably transfected with a connexin43 cDNA, Cx43-13 (high expressor of connexin43 mRNA) and Cx43-12 and Cx43-14 (intermediate expressors), were obtained from Dr. C.C.G. Naus (University of Western Ontario, London, Ont.) and used for the study (Zhu et al. 1991).

Wild type rat C6 glioma cells (American Type Culture Collection, Rockville, MD), and the connexin43 transfected clones (Cx43-13, Cx43-12, Cx43-14) were grown in DMEM (GIBCO/BRL) supplemented with 10% FBS, 25 mM HEPES, 5 U/ml penicillin and 5 mg/ml streptomycin in 5% CO_2, 95% air in humidified tissue culture incubators at 37°C. The media was changed at cell passage every 3-4 days.

2.2.3 Transfection of C6 glioma cells with an ovine IGFBP-2 cDNA

Rat C6 glioma cells were obtained from the American Tissue Culture Collection (a gift from Dr. C.C.G. Naus, University of Western Ontario, London. Ont.) and were grown in DMEM supplemented with 10% FBS, penicillin (5 U/ml), streptomycin (5 µg/ml) and gentamycin (25 µg/ml) in 5% CO_2, 95% air in humidified tissue culture incubators at 37°C. The media was changed at cell passage every 3-4 days.

Figure 2.1 Photomicrographs of primary astroglial cells identified by glial fibrillary acidic protein (GFAP) immunofluorescence. Based on cellular morphology, two populations of GFAP immunoreactive astroglia are evident: flat polygonal, protoplasmic astrocytes, and round process bearing cells (arrow), fibrous astrocytes. (A) A mixed culture of the two cell types of which the flat, polygonal astrocytes make up the majority (approximately 90%) and the round, process bearing astrocytes make up the minority (less than 10%). (B) Three flat, polygonal astrocytes and (C) a single round, process bearing astrocyte demonstrating characteristic GFAP immunofluorescence.

2


A
B
C

A 1.1 kb ovine IGFBP-2 cDNA consisting of the entire coding region (Delhanty and Han 1992) was cloned into the Hind III site of the mammalian expression vector pRc/CMV (Appendix II; Invitrogen Corporation, San Diego, CA) by Dr. P. Delhanty (Delhanty and Han 1993). The oIGFBP-2 cDNA in the antisense orientation in the pRc/CMV vector and the pRc/CMV vector were used as controls. The plasmid was transfected into C6 cells using the polybrene/DMSO technique (Aubin et al. 1991). Cells were plated on 60 mm Falcon tissue culture grade petri dishes (Becton Dickinson and Co., Franklin Lakes, NJ) at a density of 5×10^6 cells/dish in complete medium and allowed to attach overnight. The medium was aspirated and 20 µg of plasmid DNA was equilibrated with 10 µg/ml polybrene in complete medium at 37°C and added to the cells with periodic swirling for 16 h. The DNA mixture was aspirated and an equilibrated mixture of 15% dimethyl sulfoxide (DMSO) in complete medium at 37°C was added to the cells for 4.5 min at 37°C. The DMSO mixture was aspirated, the cells were washed with complete medium (2x), and allowed to recover for 24 h. Each 60 mm dish was subplated into 3 x 100 mm tissue grade petri dishes (Falcon) with selection medium (1.5 mg/ml G418 in complete medium). The cells were maintained in selection medium for 14 d which was changed every 4-5 d. Following selection, the cells were grown in complete medium and discrete colonies were picked after 4 d.

2.3 Ligand Blot Analysis

2.3.1 Collection of conditioned media

Cell cultures were incubated in serum free medium [SFM; media supplemented with 0.1 mg/ml bovine serum albumin (BSA), Sigma Chemical Co.] for 24-48 h. The medium was removed and replaced with fresh

SFM without (control) or with various doses of peptides (IGF-I, IGF-II, insulin, EGF, TGF-α, acidic FGF, basic FGF). Conditioned media were collected following treatment for the specified time interval (2-24 h), centrifuged at 1000 x *g* on a tabletop centrifuge to remove cellular debris, and the supernatant was stored at -20°C until analyzed. Cells from the same cultures were used for total RNA extraction.

2.3.2 Determination of total protein concentration

Total protein concentrations of the conditioned media were determined by the Bio-Rad Protein assay (Bio-Rad Laboratories Ltd., Richmond, CA). BSA protein standard (Bio-Rad Laboratories Ltd.) was diluted in ddH_2O to concentrations ranging between 0.1-1.4 mg/ml. Conditioned media was diluted appropriately (5-fold) in ddH_2O. Diluted dye reagent (2.5 ml) was added to duplicate 50 µl aliquots of protein standard or sample, vortexed, and allowed to sit for 30 min. The absorbance at 595 nm was determined by spectrophotometry (Model DU®-64, Beckman Instruments Inc.). The absorbance versus concentration of the protein standards was plotted graphically, and the concentration of the samples was interpolated from the standard curve.

2.3.3 Iodination of IGF-I and IGF-II

IGF-I and IGF-II were iodinated using the chloramine-T method. One µg of IGF-I or IGF-II was dissolved in 10 µl of PBS, 10 µl of 1.5 M potassium phosphate dibasic solution, pH 7.4 was added, followed by 0.5 mCi of [^{125}I]Na. A fresh solution of 0.1 mg/ml (w/v) chloramine T (N-chloro-p-toluene-sulfonamide sodium salt) in 50 mM sodium phosphate dibasic, was prepared in and added to the reaction three separate times in 3 µl volumes for 2, 1.5,

and 1 min intervals. Twenty µl of 50 mM N-acetyl tyrosine (Sigma Chemical Co.) was added and incubated for 2 min, followed by 200 µl of 60 mM potassium iodide. The reaction mixture was loaded onto a Sephadex® G-75 column (Pharmacia Biotech, Baie d'Urfe, Que.) equilibrated with column buffer (1 M acetic acid/0.1% (w/v) BSA). A further 200 µl of potassium iodide was added to the column, and subsequently eluted with column buffer into 1 ml fractions. An aliquot of 10 µl from each fraction was separated and counted on a γ-counter (model 5500 3; Beckman Instruments Inc.) to determine the peak of iodinated IGF. IGF-I and IGF-II were iodinated to a minimum specific activity of 100 µCi/µg.

2.3.4 Preparation of ligand blot

Ligand blot analysis was performed by a modification of the method described by Hossenlopp et al. (1986b). Conditioned medium (100 µl/lane; equal protein/lane) was denatured in 5x Laemmli buffer (Appendix I; Laemmli 1970) without a reducing agent, boiled for 5 min, and subjected to electrophoresis on a non-reducing 6-10% linear gradient sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE). Proteins were then transferred electrophoretically to a 0.2 µm nitrocellulose membrane (Schleicher and Schuell, Keene, NH) with a TransBlot Cell (Bio-Rad Laboratories Ltd.). The membrane was washed in 10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.5 mg/ml sodium azide, and 3% (v/v) nonidet P-40 (Sigma Chemical Co.) for 30 min at 4°C. The membrane was blocked in 0.15 M NaCl, 10 mM Tris-HCl pH 7.4, 1% (w/v) BSA for a minimum of 2 h at 4°C, followed by two 20 min washes in 0.15 M NaCl, 1 mM Tris-HCl pH 7.4, 0.1% (v/v) Tween 20. The membrane was subsequently incubated with 4×10^5 cpm of [^{125}I]IGF-II in 0.15 M NaCl, 10 mM Tris-HCl pH 7.4, 1% BSA, 0.1% Tween 20, overnight at 4°C. The

membrane was washed sequentially in 0.15 M NaCl, 10 mM Tris-HCl pH 7.4, and 0.1% Tween 20 for 2 x 15 min and 0.15 M NaCl, 10 mM Tris-HCl pH 7.4 for 3 x 15 min. The membrane was air dried and exposed to X-ray film (XAR or Biomax; Kodak Laboratories, Rochester, NY) with intensifying screens at -70°C for 3 to 7 d.

2.4 Western blot analysis

Conditioned media was electrophoresed and electroblotted as described for ligand blotting. The membrane was washed in 10 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.5 mg/ml sodium azide, and 3% nonidet P-40 for 30 min at 4°C and subsequently blocked in 4% BSA (w/v) in TTBS (Appendix I) for 1 h at RT. The membrane was washed in TTBS for 3 x 10 min followed by incubation in bovine IGFBP-2 antiserum (1:2000 dilution) in 1% BSA (w/v) in TTBS overnight at 4°C. The membrane was washed in TTBS for 3 x 10 min and incubated with secondary antibody, biotinylated goat anti-rabbit IgG (1:1000 dilution; Sigma Chemical Co.) in 1% BSA (w/v) in TTBS for 1 h at RT. Following washing in TTBS for 3 x 10 min, the membrane was incubated in ExtrAvidin®-peroxidase (1:1000 dilution; Sigma Chemical Co.) in 1% BSA (w/v) in TTBS for 1 h at RT. The membrane was washed in TTBS for 2 x 10 min and TBS (Appendix I) for 1 x 10 min and developed with 10 mg of the chromagen 3, 3'-diaminobenzidine tetrahydrochloride (DAB; Sigma Chemical Co.) in 0.05 M Tris pH 7.6. The membrane was washed and air dried.

2.5 Northern blot analysis

2.5.1 Preparation of total RNA

Total RNA was prepared from confluent cell cultures using the guanidine thiocyanate-cesium chloride method (Chirgwin et al. 1979). The conditioned media was removed and processed as described above, the cells were rinsed with ice-cold PBS, and 4 mls of guanidine thiocyanate solution (Appendix I) was added. The cells were collected using a cell scraper and transferred to a 50 ml Falcon tube. The cells were homogenized with a polytron® (model PT3000, Brinkman, Westbury, NY) at 18 000 rpm for 1 min, and subsequently centrifuged in a tabletop centrifuge (Beckman GPR, Beckman Instruments Inc.) at 900 x *g* for 10 min at room temperature. The supernatant was gently layered upon a 1.5 ml cushion of cesium chloride solution (Appendix I) and centrifuged at 1.56 x 10^5 x *g* for 16 h at 18°C. The supernatant was carefully removed, the inside of the tubes were wiped, and the pellet was dissolved in 100 µl of diethyl pyrocarbonate (DEPC) treated H_2O. An equal volume of 1:4 butanol:chloroform was added, the solution was vortexed vigorously, spun for 5 min at 2000x *g*, and the upper aqueous layer was removed and transferred to a fresh eppendorf tube. The extraction was repeated with an additional 100 µl of DEPC treated H_2O. The RNA was precipitated overnight at -20°C with the addition of 2.5x RNA volume of ice cold 100% ethanol and 1/26x RNA volume of 5 N NaCl.

RNA was pelleted at 13 500 x *g* for 30 min at 4°C, the supernatant was removed, the pellet was rinsed with ice cold 70% ethanol, spun for 10 min, and the supernatant removed. The pellet was air dried and redissolved in 50-100 µl of DEPC treated H_2O. The concentration was determined by absorbance at 260 nm by spectrophotometry (Model DU®-64, Beckman Instruments Inc.).

2.5.2 Preparation of Northern blot

Northern blotting was performed as described by Sambrook et al. (1989). Total RNA (20 µg/lane), or 3 µg of RNA ladder (0.24-9.5 kb range, GIBCO/BRL) were denatured in 1x Northern buffer (20x stock solution; Appendix I) containing 17.5% freshly deionized formaldehyde and 50% formamide for 15 min at 65°C and subjected to electrophoresis in a 1% agarose gel containing 6.6% formaldehyde. The RNAs were transferred to a Zeta-probe® GT nylon membrane (Bio-Rad Laboratories) by the capillary transfer technique (Southern 1975). Following transfer, the RNA ladder was cut from the membrane and stained in 0.2% methylene blue (Sigma Chemical Co.) for 30 sec, destained in water, and the RNA bands were marked for future reference. The membrane was washed in 10x SSC (20x stock solution; Appendix I) for 10 min x 2, then UV cross-linked (Stratalinker®, Stratagene Inc., LaJolla, CA), and baked in a vacuum oven (Napco® Model 5831, Napco Scientific Co., Tualatin, OR) for 1 h at 75°C.

2.5.3 Preparation of [^{32}P]cDNA probes

The 585 bp rat IGFBP-2 cDNA, (a gift from Dr. M. Rechler, NIH, Bethesda, MD), was subcloned into pGEM-3 (Promega Corporation, Madison, WI). The 2.0 kb IGFBP-3 cDNA (a gift from Dr. A. Herington, University of Melbourne, Melbourne, Australia) was subcloned into pGEM4Z (Promega Corp.). The 444 bp rat IGFBP-4, the 300 bp IGFBP-5, and the 246 bp IGFBP-6 cDNAs (gifts from Dr. S. Shimasaki, The Whittier Institute, San Diego, CA) were obtained in pBluescript SK$^+$ (Stratagene Inc.). The 500 bp rat IGF-I cDNA (a gift from Dr. L. Murphy, University of Manitoba, Winnipeg, Man.) was subcloned into pGEM Blue (Promega Corp.), and the 1413 bp mouse IGF-II cDNA (a gift from Dr. G. Bell, University of Illinois, Chicago, IL) was

subcloned into pGEM 4Z (Promega Corp.). The 1.4 kb rat connexin43 cDNA (a gift from Dr. E. Beyer, Washington University, St. Louis, MO) was obtained from Dr. C.C.G. Naus, University of Western Ontario, London, Ont.

The plasmids were digested with the appropriate restriction enzymes and electrophoresed on a 0.1% agarose gel. The linearized insert was cut out of the gel and purified using the Geneclean kit (Bio/Can Scientific, Mississauga, Ont.). The concentration of purified insert was determined by spectophotometry.

The cDNA inserts were labeled to specific activities of 1-2 x 10^9 cpm/µg by the random priming technique using an Oligo-labeling kit (Pharmacia Canada Inc.). One hundred ng of cDNA insert was denatured by boiling for 5 min, immediately quenched on ice for 2 min, and incubated for 1 h at 37°C with reagent mix, 5 µCi of [α-^{32}P]dCTP (3000 Ci/mmol; ICN), and 1 µl of the Klenow fragment of DNA polymerase I. Unincorporated [α-^{32}P]dCTP was removed by running the reaction sample through an equilibrated Sephadex®-G50 Nick™ column (Pharmacia Biotech) with 400 µl of TE buffer pH 7.4 (Appendix I). Radiolabeled cDNA inserts were eluted from the column by an additional 400 µl of TE buffer. A 4 µl aliquot was placed in 5 ml of ScintiSafe™ Econo1 (Fisher Scientific, Nepean, Ont.) and counted in a β-counter (model LS 5000TD; Beckman Instruments).

2.5.4 Hybridization, washing and autoradiography

Blots were prehybridized with 20 ml of Northern hybridization buffer (Appendix I) for a minimum of 2 h at 42°C in a rotating hybridization incubator (model 310; Robbins Scientific Co., Sunnyvale, CA). Blots were then hybridized with [^{32}P]IGFBP cDNA probes at a specific activity of 2 x 10^6 cpm/ml hybridization buffer at 42°C overnight.

The blots were washed in 1x SSC/0.1% SDS at 42°C for 30 min twice, and in 0.1x SSC/0.1% SDS at 42°C for 30 min twice, air dried and exposed to XAR film (Kodak Laboratories) using intensifying screens at -70°C. The blots were stripped in between hybridizations by washing in 0.01x SSC/0.5% SDS for 30 min each at 80°C .

Consistency in loading and transfer of total RNAs in each lane was checked by probing the blots with a radiolabeled cDNA for 18S ribosomal RNA (a gift from Dr. David Denhardt, Rutgers University, Piscataway, NJ). The autoradiograms were quantified by densitometry. The relative densities of the bands were expressed as arbitrary absorbance units. To correct for minor differences in loading of total RNA in Northern blots, a ratio of the relative density of each specific band with the relative density of the 18S ribosomal RNA band was calculated before comparisons were made.

2.6 Slot blot analysis

Total RNAs (10 µg) were denatured in 6.15 M formaldehyde/10x SSC at 65°C for 15 min. Samples were applied in triplicate, onto Zeta-probe®GT membrane (Bio-Rad Laboratories) using the Minifold II Slot-Blot system (Schleicher & Schuell). The wells were washed with 10x SSC, the membrane then washed in 10x SSC for 10 min, UV cross-linked, and baked at 80°C for 1 h. The slot-blot was hybridized with [^{32}P]IGFBP cDNAs and washed as described for Northern blotting.

2.7 Affinity cross-linking

Cells were plated at a density of 3 x 10^5 cells in poly-L-lysine (0.05 mg/ml) coated 6-well tissue culture plates in complete medium. Upon reaching confluence at 48 h, the cell monolayers were washed in HEPES

binding buffer pH 7.4 (Appendix I) and subsequently incubated with 5 x 10^5 cpm of [^{125}I]IGF-I or IGF-II with or without competitors (IGF-I, IGF-II, insulin, heparin, GRGDSP) in HEPES binding buffer for 6 h at 4°C. The incubating solution was aspirated and proteins were cross-linked by incubating 0.1 mM disuccinimidyl suberate (DSS; Pierce, Rockford, IL), solubilized in DMSO, in cross-linking buffer (Appendix I) for 30 min at RT. The cross linking solution was aspirated and the proteins were solubilized by the addition of 1x Laemmli buffer. The samples were denatured by boiling and run on 3-14% gradient SDS-PAGE. The gels were fixed in 25% methanol, 10% acetic acid in water, dried on a gel dryer (model 583; Bio-Rad Laboratories Ltd.), and exposed to XAR-5 or Biomax film (Kodak Laboratories) for autoradiography.

2.8 Immunoprecipitation

Conditioned medium was incubated with 2 x 10^5 cpm of [^{125}I]IGF-II for 1 h at 4°C and subsequently cross-linked with the addition of 10 mM DSS. Cell monolayers were cross-linked with [^{125}I]IGF-II as described above, solubilized in membrane homogenization buffer (Appendix I), and sonicated for 3 x 10 s bursts with a membrane sonicator (model 300; Fisher Scientific). The resulting cell suspensions and conditioned media were incubated with antiserum against bovine IGFBP-2 at 1:50 dilution at 4°C overnight on a rotating wheel (model WB10; Robbins Scientific Co.). Immune complexes were precipitated with the addition of protein A-Sepharose® (Pharmacia Biotech). Immune complexes were pelleted by centrifugation at 13 500 x *g* for 10 min, and washed twice with membrane homogenization buffer. Protein complexes were released by the addition of 1x Laemmli buffer, boiled, and run on 6-14% gradient SDS-PAGE. The gels were dried and exposed to Biomax film for autoradiography.

2.9 IGF-I radioimmunoassay (RIA)

Conditioned media (5 ml) was lyophilized, solubilized in 1 M acetic acid at 4°C overnight, and applied to an equilibrated and calibrated Sephadex® G-50 (Pharmacia Biotech) column. IGFs were separated from IGFBPs by elution with 0.2 M acetic acid. The IGF containing fractions were pooled, and dried in a speed vacuum (model SVC1000; Savant Instruments Inc., Farmingdale, NY). The samples were assayed for IGF-I according to the method described by Furlanetto et al. (1977). IGF-I standards or acid extracted conditioned media samples were incubated in duplicate at room temperature in a neutral phosphate buffer with a 1:3000 dilution of IGF-I antiserum. After 4 h, 1 x 10^4 cpm of [^{125}I]IGF-I were added, incubation was continued at 4°C for 16-18 h. Bound and free IGF were separated by double antibody immunoprecipitation with anti-rabbit immunoglobulin.

2.10 Analysis of growth

Selected clones were plated at a density of 1 x 10^5 cells in T-25 tissue culture flasks (Falcon) in complete medium. Cell number was determined in triplicate, the following day (time zero), and every 24 h thereafter, for a period of 72 h on a Coulter counter® (model Zf; Coulter Electronics Inc., Hialeah, FL). Growth curves were constructed by plotting mean cell number against time. The doubling time (Dt) for each clone was calculated from the linear portion of the curve when expressed in a logarithmic scale by the following equation.

Dt=change in time/log(change in cell number)

Each experiment was performed three times and the mean ± SEM doubling time was calculated. Each clone was compared to the wild type C6 cells using analysis of variance.

For growth curves in reduced serum, clones were plated at a density of 2×10^5 cells in 6-well or 5×10^4 cells in 24-well tissue culture plates in complete medium. On the following day, the cells were rinsed and placed in medium containing 1% FBS with or without peptides. The peptides were replenished on a daily basis.

2.11 *In situ* hybridization

Radiolabeled anti-sense and sense IGFBP-2 and IGFBP-3 cRNAs were transcribed using the Riboprobe Gemini II core system (Promega Corp.) and [^{35}S]UTP (ICN). Fragments of the cRNAs of approximateiy 150 bp were generated by alkaline digestion in 0.2 M carbonate buffer pH 10.2 (Appendix I).

Purified astroglia cultures were plated at a density of 2×10^5 cells/ml onto 8-well Tissue-Tek chamber slides. Following two days of culture in BME with 10% FBS, the medium was changed to SFM. After 48 h, IGF-I (200 ng/ml), IGF-II (200 ng/ml) and insulin (1 µg/ml) were added, and the cells were further incubated for 24 h. The cells were then fixed in 4% paraformaldehyde and 0.2% glutaraldehyde for 30 min, and permeabilized with 0.2% Triton-X in PBS for 15 min at RT. Following 2x 2 min washes in PBS, the slides were incubated in acetic anhydride:0.1 M triethanolamine buffer (Sigma Chemical Co.) for 10 min at RT, washed in PBS, dehydrated in ascending ethanol series (70%, 90%, 100%) and air dried. The chamber grids were then removed from the slides.

The slides were prehybridized with *in situ* hybridization buffer (Appendix I) at 42°C for 2 h. Hybridization was performed with specific riboprobes (1×10^6 cpm/slide) for overnight at 42°C. The slides were then incubated with hybridization buffer for 10 min at 65°C followed by treatment with 20 µg/ml of RNAse A (Pharmacia Biotech) for 30 min at 37°C to reduce

background. The slides were washed sequentially in 2x SSC at RT (30 min x 2), 2x SSC at 42°C (30 min x 4) and in 0.1x SSC at RT (15 min x 2). Slides were dehydrated in ascending ethanol series, air dried and coated with NTB-3 nuclear track emulsion (Kodak Laboratories) and exposed for 14 d at 4°C. Slides were developed in D19 (Kodak Laboratories) for 1 min, fixed in Kodafix for 10 min, counterstained with Harris' haematoxylin (Fisher Scientific) and eosin (Fisher Scientific), dehydrated and coverslipped with Permount (Fisher Scientific).

2.12 [^{3}H]thymidine incorporation

Cells were subplated on to 48 well plates (Costar Corporation), at a density of 3 x 10^4 cells/well for C6 and 2 x 10^4 cells/well for Cx43-13 cells, and cultured to subconfluence for 48 h. The cells were incubated in SFM for 24 h and then exposed to either IGF-I or -II (25, 50, 100, 250, 500 ng/ml) or CM from clone Cx43-13 [10, 25, 50, 75, 100% (v/v)] in varying doses. After incubation for 10 h, [methyl-^{3}H]thymidine 0.5 µCi/well (1.0 µCi/ml, Amersham Canada, Oakville, Ontario) was added for a further 2 h. The reaction was stopped by aspirating the incubating solution and washing with cold Hanks Balanced Salt Solution (HBSS, Sigma Chemical Co.). The cultures were then incubated with 10% (w/v) trichloroacetic acid (TCA, Sigma Chemical Co.) at RT for 10 min. The TCA solution was aspirated and the cultures were then incubated with 12.5% (w/v) TCA for 2 min. The TCA was removed, and the cells were solubilized in 0.5 ml 0.1 N sodium hydroxide. The solution was added to 5 ml of scintillation cocktail (ScintiVerse^{TM}, Fisher Scientific) and counted in a β-scintillation counter. Optimal cell density and the time of maximum thymidine incorporation were determined for each cell line prior to analysis.

2.13 Immunocytochemistry

C6 glioma and Cx43-13 cells were cultured on glass coverslips coated with poly-L-lysine, fixed in 95% ethanol/5% glacial acetic acid (v/v) for 20 min at -20°C, rinsed with PBS, and incubated for 2 h in a 1:500 dilution of rabbit polyclonal connexin43 antiserum raised against a synthetic peptide corresponding to amino acids 302-319 of the predicted amino acid sequence (a gift from Dr. B. Nicholson, SUNY, Buffalo, NY). Control slides were incubated in a preimmune serum (1:500) from the same rabbit. This was followed by incubation in fluorescein-conjugated goat antirabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:50. Coverslips were then rinsed in PBS and mounted on slides in PBS containing 50% (v/v) glycerol and 0.1% *p*-phenylenediamine.

CHAPTER THREE

ASTROGLIAL INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS ARE REGULATED BY IGFs AND INSULIN

3.1 INTRODUCTION

The IGFs have been implicated as important molecules contributing to the growth and differentiation of the brain. IGFs are produced during development of the brain with specific regional and temporal patterns of expression that correlate with the growth and differentiation of specific cell types (Andersson et al. 1988, Rotwein et al. 1988, Wood et al. 1990, Ayer-Le Lievre et al. 1991, Bach et al. 1991, Lee et al 1992, 1993). Certain IGFBPs have distinct spatio-temporal patterns of expression during development of the brain suggesting that locally produced IGFBPs are important in regulating IGF biological activity (Tseng et al. 1989, Wood et al. 1992, Lee et al. 1992, Bach and Bondy 1992, Lee et al. 1993, Bondy and Lee 1993, Brar and Chernausek 1993, Sullivan and Feldman 1994).

During development, astroglia provide a scaffolding for the migration of neurons from the proliferative ventricular surface of the neural tube to outer regions of the CNS. They are a source of trophic factors for neurons and aid in maintaining cellular homeostasis by buffering the extracellular environment. IGFs, their receptors, and IGFBPs have been localized to astroglial cells in various regions during brain development. Astroglial cells cultured from newborn rats synthesize IGF-I, IGF-II, both receptors, in addition to IGFBPs (Han et al. 1987b, 1988b, Ocrant et al. 1989, 1990, Olson et al. 1991).

Numerous studies suggest that both short and long term biological actions of IGFs in many tissue/cell systems may be modulated by alterations in the levels of IGFBPs in the extracellular *milieu* (McCusker and Clemmons 1992). Therefore, the regulation of the synthesis of IGFBPs would play an important role in modulating the biologic actions of IGFs on astroglia or

other neighbouring cell types and would potentially have important consequences on the growth and differentiation of developing brain tissues. We hypothesized that astroglial IGFBPs are regulated by growth factors produced locally in the brain including the IGFs.

3.2 OBJECTIVE

The objective of this study was to determine whether IGF-I, IGF-II and insulin regulate the biosynthesis of IGFBPs in primary cultures of newborn rat astroglia.

3.3 METHODS

3.3.1 Analysis of secreted IGFBPs

Conditioned media were collected from confluent cultures in T-75 flasks following 24 h incubation with either IGF-I (10, 100, 200 ng/ml), IGF-II (10, 100, 200 ng/ml) or insulin (0.1, 1.0, 10 μg/ml). These peptide concentrations were within the dose range that was observed to stimulate thymidine incorporation in this cell type (Han et al. 1987). The lower doses of IGFs and insulin reflect the physiologic concentrations that exist in tissue fluids, and the higher doses reflect pharmacologic concentrations required to stimulate a biologic response. Ligand blot analysis was performed as described in section 2.3 using [^{125}I]IGF-II as a radiolabel. For the time course studies, conditioned media were collected at the various time intervals indicated.

3.3.2 Analysis of IGFBP stable mRNA levels

Confluent cells in T-75 flasks were used for extraction of total RNA and subjected to Northern blot and slot blot analyses as described in section 2.5. Changes in IGFBP stable mRNA levels were quantified by laser densitometry (Ultrascan XL, LKB, Bromma, Sweden) of the autoradiograms of slot blots. The relative densities of the bands were expressed as arbitrary absorbance units (au). To correct for minor differences in loading of total RNA, a ratio of the relative density of each specific band with the relative density of the 18S ribosomal band was calculated before comparisons were made.

3.3.3 Cellular localization of IGFBP mRNAs

In situ hybridization was performed as described in section 2.11 to determine the cellular localization of IGFBP mRNAs.

3.3.4 Statistical analysis

Analysis of variance was used to determine statistically significant changes in IGFBP protein and mRNA levels.

3.4 RESULTS

3.4.1 Astroglial IGFBPs are increased by IGF-I and IGF-II

Two binding proteins of M_r 34 kDa and 40-43 kDa were detected in astroglial conditioned media by Western ligand blot analysis with either [^{125}I]IGF-I or [^{125}I]IGF-II. Both BPs exhibited greater affinity for [^{125}I]IGF-II as demonstrated by more intense IGFBP bands when the two radiolabeled IGFs were used under equivalent conditions (data not shown). Subsequent ligand

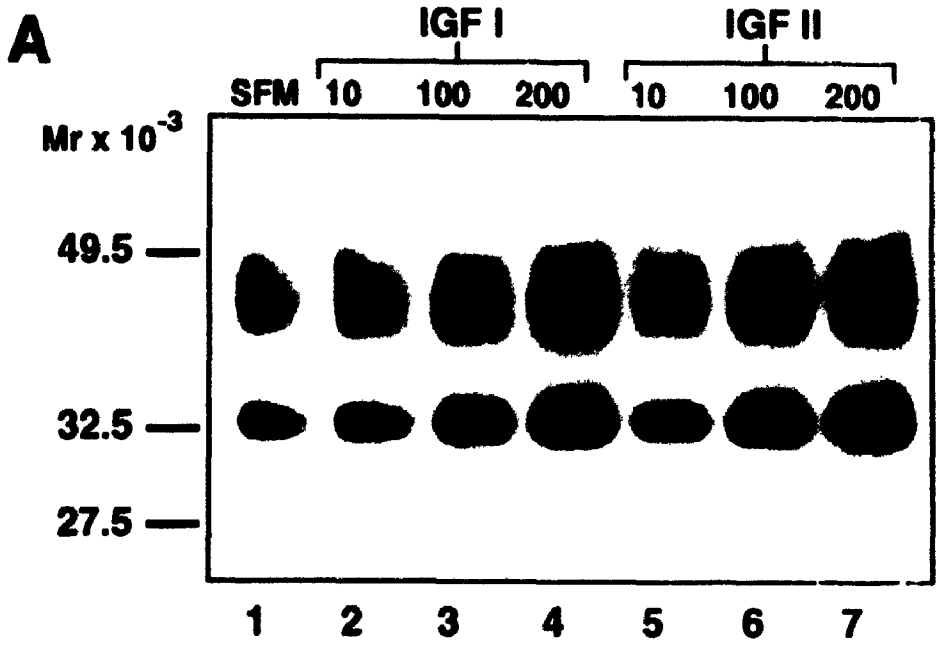
blots were therefore performed with [^{125}I]IGF-II as a radioligand. A representative ligand blot of conditioned media obtained from astroglial cultures treated with various doses of IGF-I and IGF-II is shown in Figure 3.1 A. The 34 kDa IGFBP was shown immunologically to be rat IGFBP-2. The 40-45 kDa IGFBP appeared as a broad band, possibly due to glycosylation. This finding and our detection of IGFBP-3 mRNA from astroglia (see below) suggested that the 40-45 kDa IGFBP was IGFBP-3.

Densitometric analysis was used to quantitate the relative changes in IGFBP levels by ligand blotting. Ligand blotting is a semi-quantitative technique and therefore the changes in IGFBP levels are expressed relative to the control conditions in SFM. Densitometric analysis of three separate experiments revealed that both IGF-I and IGF-II increased IGFBP-2 and IGFBP-3 in the conditioned media in a dose dependent manner. A maximal 2-fold increase in IGFBP-2 protein over control with IGF-I 200 ng/ml and a 3-fold increase with IGF-II 100 ng/ml was observed (Figure 3.1 B). IGFBP-3 was increased over 3-fold by both IGF-I and IGF-II at 200 ng/ml (Figure 3.1 C).

3.4.2 Astroglial IGFBPs are increased by insulin

Insulin, at higher concentrations (1 and 10 μg/ml), increased both IGFBP-2 and IGFBP-3 in the conditioned media (Figure 3.2 A). Densitometric analysis of three separate experiments showed a maximum stimulation of slightly over 2-fold at an insulin concentration of 1 μg/ml (Figure 3.2 B). IGFBP-3 protein levels were also increased in a dose dependent manner with a maximal stimulation of 5-fold over control at a concentration of 10 μg/ml (Figure 3.2 C). Although the changes in IGFBP-2 and IGFBP-3 in response to insulin were not significant from control conditions, a similar trend was observed in all three experiments.

Figure 3.1. Ligand blot analysis of conditioned media (CM) from astroglia treated with IGF-I and IGF-II. Conditioned media were collected from primary astroglia treated with IGF-I or IGF-II for 24 h, subjected to SDS-PAGE, transferred to nitrocellulose and incubated with [^{125}I]IGF-II. (**A**) Autoradiograph of a representative ligand blot showing, lane 1: SFM control, lanes 2-4: IGF-I treatment (10, 100 and 200 ng/ml), and lanes 5-7: IGF-II treatment (10, 100 and 200 ng/ml). (**B**) The densitometric analysis (three separate experiments) of changes in IGFBP-2 levels in the CM from astroglia treated with either IGF-I or IGF-II expressed as mean $\pm$ SEM of percent change from control. (C) A similar analysis for IGFBP-3. * indicates a statistically significant change ($p < 0.05$).


A
IGF I
IGF II
SFM
10
100
200
10
100
200
Mr x 10-3
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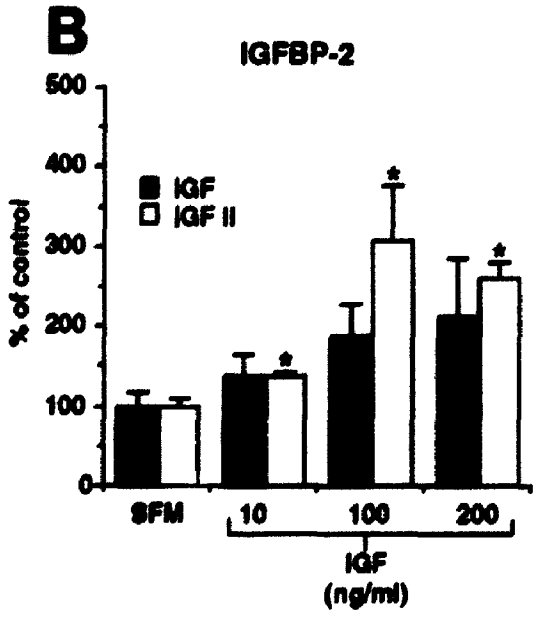
32.5
27.5
1
2
3
4
5
6
7


B
IGFBP-2
% of control
500
400
300
200
100
0
IGF
IGF II
SFM
10
100
200
IGF
(ng/ml)

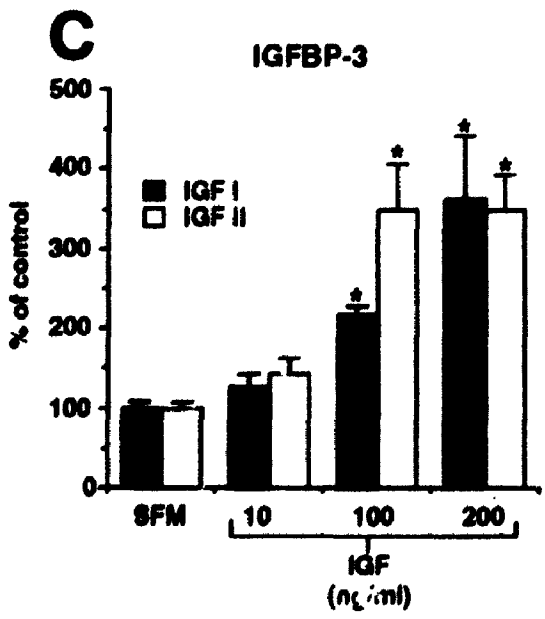
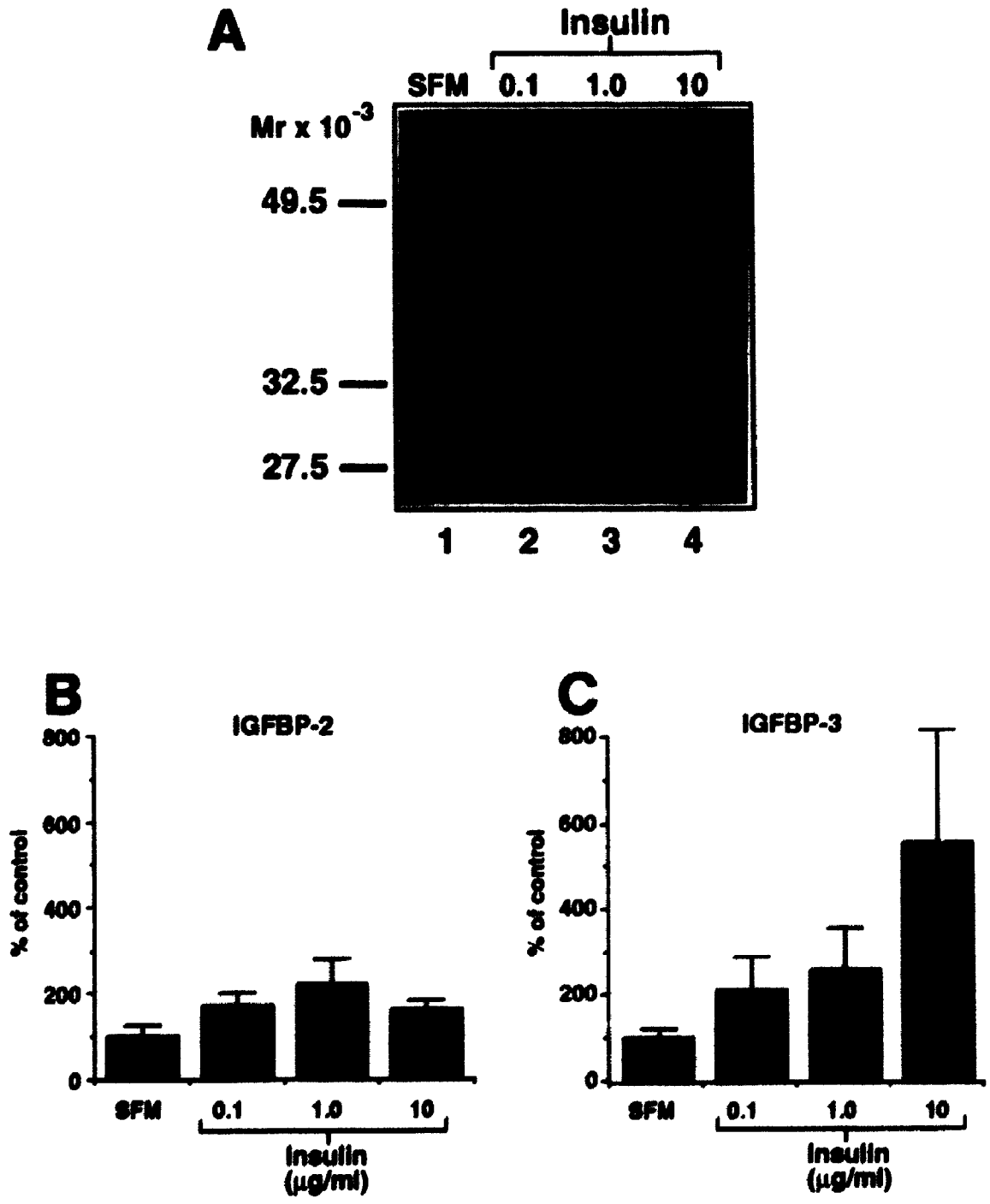

C
IGFBP-3
% of control
500
400
300
200
100
0
IGF I
IGF II
SFM
10
100
200
IGF
(ng/ml)

Figure 3.2. Ligand blot analysis of CM from astroglia treated with insulin. **(A)** Autoradiograph of a representative ligand blot showing, lane 1: SFM control, lanes 2-4: insulin treatment (0.1, 1.0 and 10 μg/ml). **(B)** The densitometric analysis (three separate experiments) of changes in IGFBP-2 levels in the CM from astroglia treated with varying doses of insulin and expressed as mean ± SEM of percent change from control. **(C)** A similar analysis for IGFBP-3.


A
Insulin
SFM
0.1
1.0
10
Mr x 10-3
49.5
32.5
27.5
1
2
3
4
B
IGFBP-2
% of control
800
600
400
200
0
SFM
0.1
1.0
10
Insulin
(µg/ml)
C
IGFBP-3
% of control
800
600
400
200
0
SFM
0.1
1.0
10
Insulin
(µg/ml)

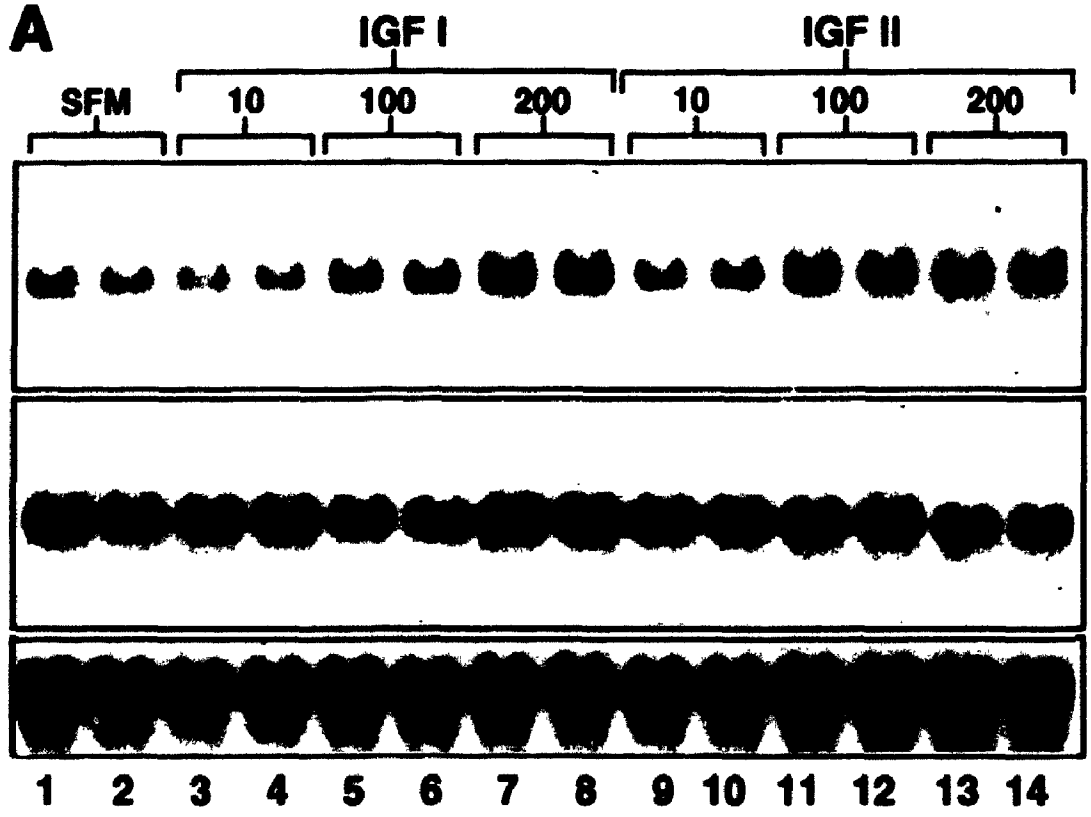
3.4.3 The effect of IGF-I and IGF-II on IGFBP stable mRNA levels

To determine whether the increase in synthesis of IGFBP-2 and IGFBP-3 were due to an increase in stable mRNA, Northern blot analysis was performed on astroglial cultures treated with the same doses of IGF-I and IGF-II. Figure 3.3 A is a representative Northern blot of astroglial total RNA probed sequentially with radiolabeled IGFBP-2 and IGFBP-3 cDNAs. Upon hybridization with IGFBP-2 and IGFBP-3 cDNAs, transcripts of 1.6 kb and 2.4 kb respectively were observed. Stable IGFBP-2 mRNA levels increased upon treatment with IGF-I and IGF-II in a dose dependent manner corresponding to their effect on IGFBP-2 protein levels. Densitometric analysis of a corresponding slot blot showed a 2-fold stimulation of IGFBP-2 mRNA levels in response to IGF-I at 200 ng/ml (Figure 3.3 B). IGF-II at 100 ng/ml maximally stimulated IGFBP-2 stable mRNA levels 2-fold. Stable IGFBP-3 mRNA levels remained unchanged in contrast to the stimulation of IGFBP-3 protein levels (Figure 3.3 A). Densitometric analysis demonstrated that IGFBP-3 mRNA levels did not increase in response to either IGF-I or IGF-II (Figure 3.3 C). Similar results were observed with two additional experiments.

3.4.4 The effect of insulin on IGFBP stable mRNA levels

Insulin increased IGFBP-2 stable mRNA levels but not IGFBP-3 stable mRNA levels. This finding is similar to that observed with IGF-I or IGF-II treatment (Figure 3.4 A). Densitometric analysis of a corresponding slot blot showed that insulin increased IGFBP-2 stable mRNA levels from two-fold at 0.1 µg/ml to 2.5-fold at 1.0 and 10 µg/ml (Figure 3.4 B). The increase in IGFBP-2 stable mRNA levels corresponded to the increase in IGFBP-2 observed by ligand blotting. In contrast, IGFBP-3 stable mRNA levels were

Figure 3.3. Northern blot analysis of total RNA from astroglia treated with IGF-I and IGF-II, and probed sequentially with [^{32}P]labeled rat IGFBP-2 (upper panel), IGFBP-3 (middle panel), and 18S ribosomal (lower panel) cDNAs. Total RNA was extracted from the same primary cultures from which CM was collected for IGFBP analysis. (A) A representative Northern blot of total RNA from cultures treated for 24 h with SFM (lanes 1 and 2), IGF-I 10 ng/ml (lanes 3 and 4), IGF-I 100 ng/ml (lanes 5 and 6), IGF-I 200 ng/ml (lanes 7 and 8), IGF-II 10 ng/ml (lanes 9 and 10), IGF-II 100 ng/ml (lanes 11 and 12), and IGF-II 200 ng/ml (lanes 13 and 14). (B) The densitometric analysis of the corresponding slot blot of total RNAs (each sample analyzed in triplicate) for IGFBP-2 mRNA, and expressed as mean ± SEM of percent change from control (cultures treated with SFM). (C) A similar analysis for IGFBP-3. * indicates a statistically significant change ($p < 0.05$). Similar results were observed in two additional experiments.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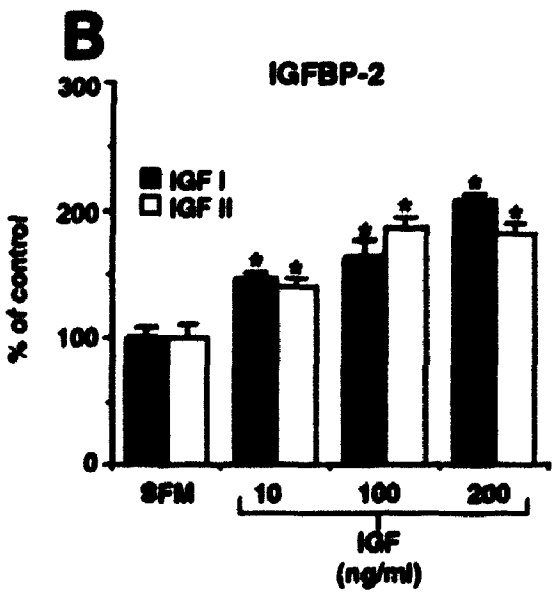
IGF I
IGF II
SFM
10
100
200
10
100
200
1 2 3 4 5 6 7 8 9 10 11 12 13 14


B
IGFBP-2
300
200
100
0
% of control
IGF I
IGF II
SFM
10
100
200
IGF
(ng/ml)

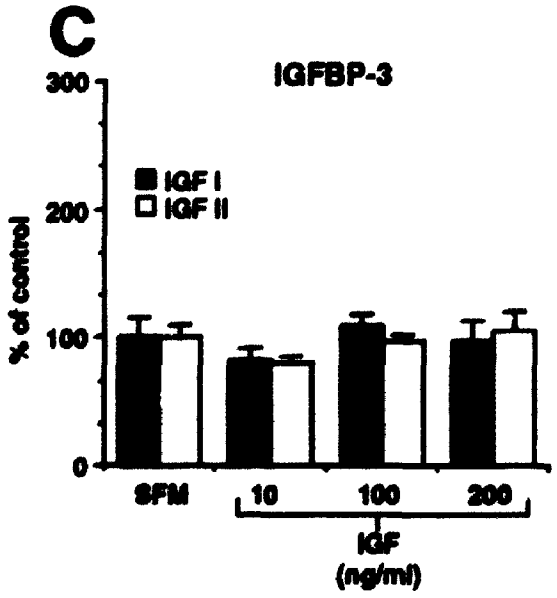
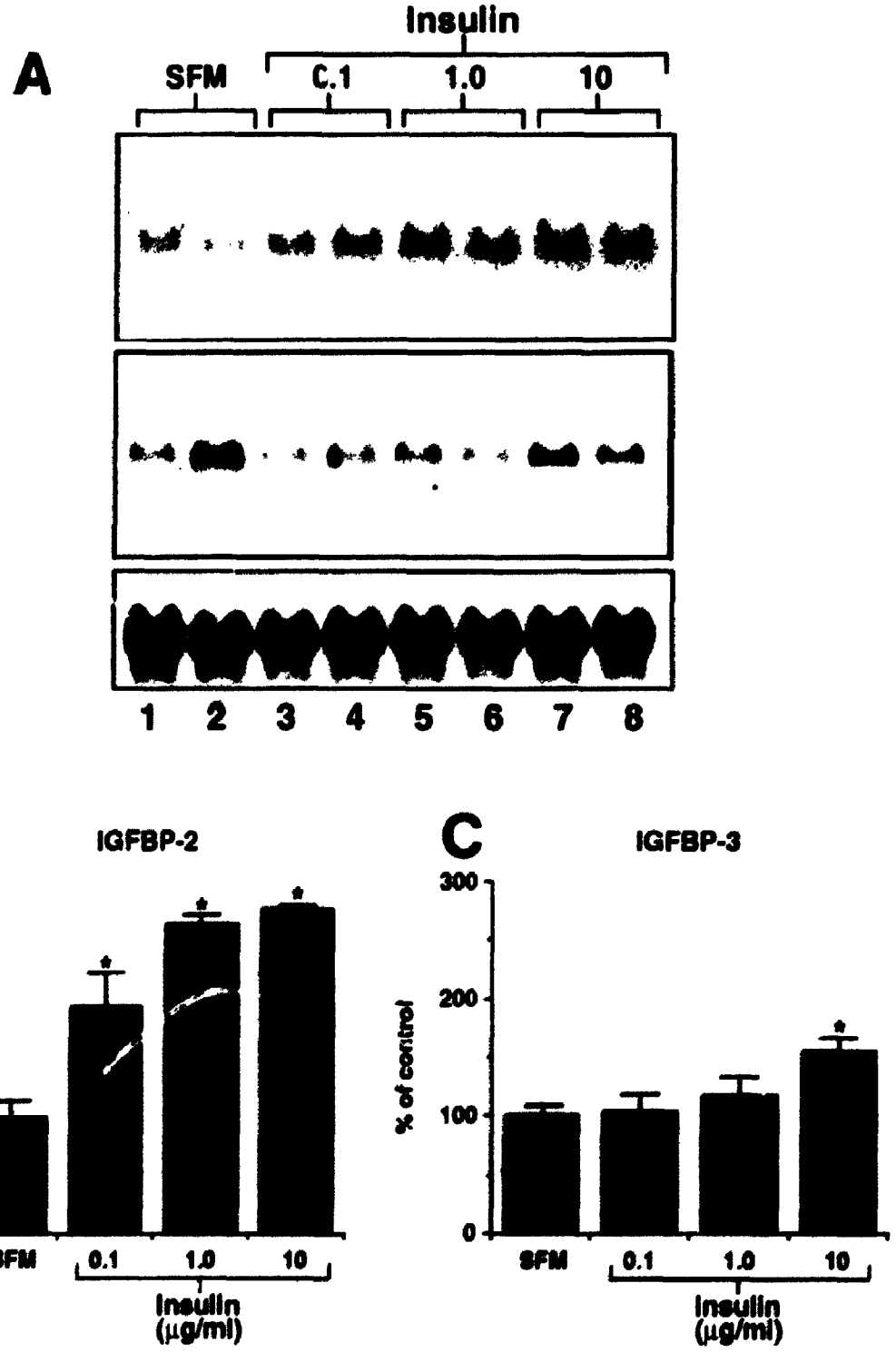

C
IGFBP-3
300
200
100
0
% of control
IGF I
IGF II
SFM
10
100
200
IGF
(ng/ml)

Figure 3.4. Northern blot analysis of total RNA prepared from astroglia treated with insulin and probed sequentially with [^{32}P]labeled rat IGFBP-2 (upper panel), IGFBP-3 (middle panel), and 18S ribosomal (lower panel) cDNAs. (A) A representative Northern blot of total RNA from cultures treated for 24 h with SFM (lanes 1 and 2), insulin 0.1 μg/ml (lanes 3 and 4), insulin 1.0 μg/ml (lanes 5 and 6), and insulin 10 μg/ml (lanes 7 and 8). (B) The densitometric analysis of the corresponding slot blot of total RNAs (each sample analyzed in triplicate) for IGFBP-2 mRNA, and expressed as mean ± SEM of percent change from control (cultures treated with SFM). (C) A similar analysis for IGFBP-3. * indicates a statistically significant change ($p < 0.05$). Similar results were observed in two additional experiments.


A
Insulin
SFM
C.1
1.0
10
1 2 3 4 5 6 7 8
B
IGFBP-2
% of control
300
200
100
0
SFM
0.1
1.0
10
Insulin
(µg/ml)
C
IGFBP-3
% of control
300
200
100
0
SFM
0.1
1.0
10
Insulin
(µg/ml)

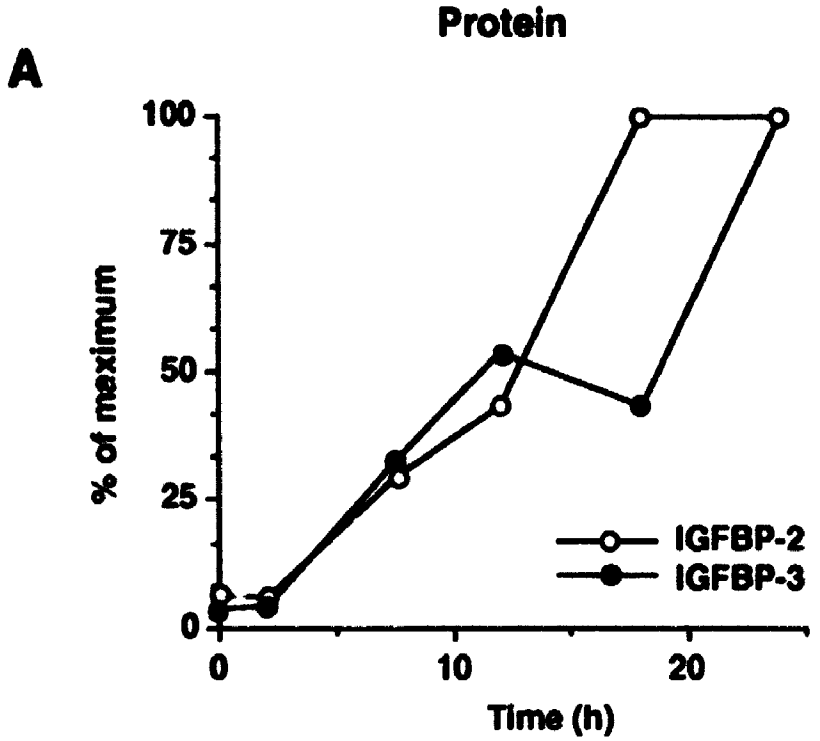
not altered from control with insulin treatment except at a high dose of 10 µg/ml (Figure 3.4 C). This finding was observed in two additional experiments.

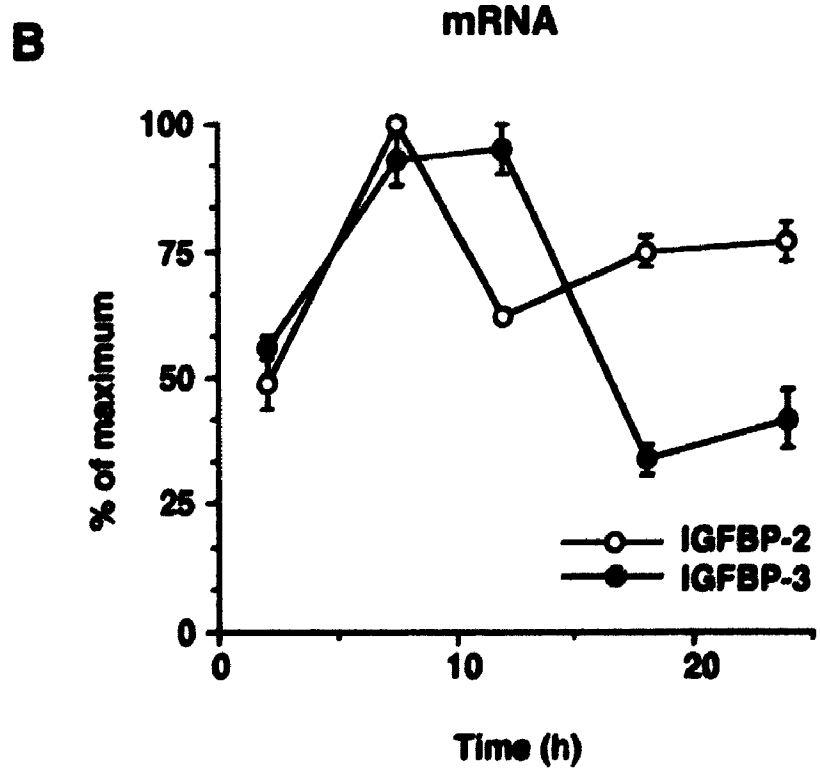
3.4.5 The time course of IGF-I induction of IGFBP mRNA and protein

Purified astroglia cultures in T-75 flasks were serum starved for 48 h and subsequently treated with IGF-I (200 ng/ml). Cultures were stopped at time points of 2, 7.5, 12, 18 and 24 h. Conditioned media were collected and analyzed by ligand blotting. Cells from the same flasks were harvested, total RNA was extracted and subjected to Northern and slot blot analyses as described in Sections 2.2.7-2.2.8. IGFBP protein and mRNA levels were normalized as percentages of maximum levels to allow for time course comparisons.

IGFBP-2 protein levels in the conditioned media increased slowly over time to reach maximum levels by 18 h and remained at this level over 24 h (Figure 3.5 A). IGFBP-3 protein levels increased similarly to reach maximum levels by 24 h. IGFBP-2 and IGFBP-3 stable mRNA levels showed a different time course response to IGF-I. IGFBP-2 stable mRNA increased rapidly after the addition of IGF-I 200 ng/ml, reaching maximum levels by 7.5 h and were maintained at higher levels but below the maximum response between 12 and 24 h (Figure 3.5 B). IGFBP-3 stable mRNA levels were increased rapidly by IGF-I treatment, reaching maximum levels by 12 h and decreased to control levels by 18 and 24 h. A rapid but shorter duration of increase in IGFBP-3 mRNA levels by IGF-I may partly explain the non-corresponding changes in IGFBP-3 protein and mRNA levels at 24 h.

Figure 3.5. Time course of IGF-I induced increase in IGFBP-2 and IGFBP-3 (**A**) protein in the conditioned media, and (**B**) total stable mRNA levels. Primary astroglia were treated with IGF-I 200 ng/ml and the incubation was stopped at various time points (2, 7.5, 12, 18 and 24 h). Conditioned media were analyzed by ligand blotting, quantified by densitometry and the results were expressed as percent of maximum response for a single experiment (**A**). Total RNA was analyzed by slot blotting (triplicate samples), quantified by densitometry and expressed as mean ± SEM of percent of maximum response (**B**).


A
Protein
100
75
50
25
0
% of maximum
0
10
20
Time (h)
IGFBP-2
IGFBP-3


B
mRNA
100
75
50
25
0
% of maximum
0
10
20
Time (h)
IGFBP-2
IGFBP-3

3.4.6 The IGFBP stable mRNA levels are increased by IGF-I at 12 h

To determine if IGFBP-2 and IGFBP-3 stable mRNA levels were increased by IGF-I in a dose dependent manner at 12 h (maximum response as observed by the time course experiment), Northern blot analysis was performed on total RNA from cells treated with varying doses of IGF-I for 12 h (Figure 3.6 A). Both IGFBP-2 and IGFBP-3 stable mRNA levels were increased in a dose dependent manner. Densitometric analysis of the Northern blot demonstrated that IGFBP-2 stable mRNA levels were increased 2.5 fold from control at doses of IGF-I 100 to 200 ng/ml (Figure 3.6 B). IGFBP-3 stable mRNA were increased 1.5 fold from control by IGF-I (100 and 200 ng/ml) demonstrating that IGFBP-3 stable mRNA is increased by IGF-I at 12 h (Figure 3.6 C).

3.4.7 Analysis of the stability of IGFBP-2 mRNA

To determine if the increase in IGFBP-2 mRNA levels at 24 h by IGF-I was due to an increase in mRNA stability, purified astroglia cultures were serum starved for 48 h and incubated without or with IGF-I 200 ng/ml. Twenty-four hours later, actinomycin-D (5 μg/ml) was added to both IGF-I treated and control cells, and total RNA was isolated at time points 2, 4 and 6 h. IGFBP-2 mRNA was quantified by slot blot analysis as described in Section 2.2.8. A ratio of IGFBP-2 mRNA to 18S rRNA was calculated prior to analysis.

IGFBP-2 stable mRNA levels decreased over time in both control and IGF-I conditions (Figure 3.7). The slopes of the control and IGF-I treated group were compared using analysis of covariance and were found to be statistically not different, suggesting that IGF-I treatment did not affect the stability of IGFBP-2 mRNA.

Figure 3.6. Northern analysis of total RNA from astroglia treated with varying doses of IGF-I (10, 100 and 200 ng/ml) for 12 h and probed sequentially with [^{32}P]labeled rat IGFBP-2 (upper panel), IGFBP-3 (middle panel), and 18S ribosomal (lower panel) cDNAs. (**A**) A representative Northern blot of total RNA from astroglia treated with SFM (lane 1), IGF-I 10 ng/ml (lane 2), IGF-I 100 ng/ml (lane 3), and IGF-I 200 ng/ml (lane 4). The changes in stable mRNA levels were quantified by densitometry of the above Northern blot and presented separately for IGFBP-2 (**B**) and IGFBP-3 (**C**).

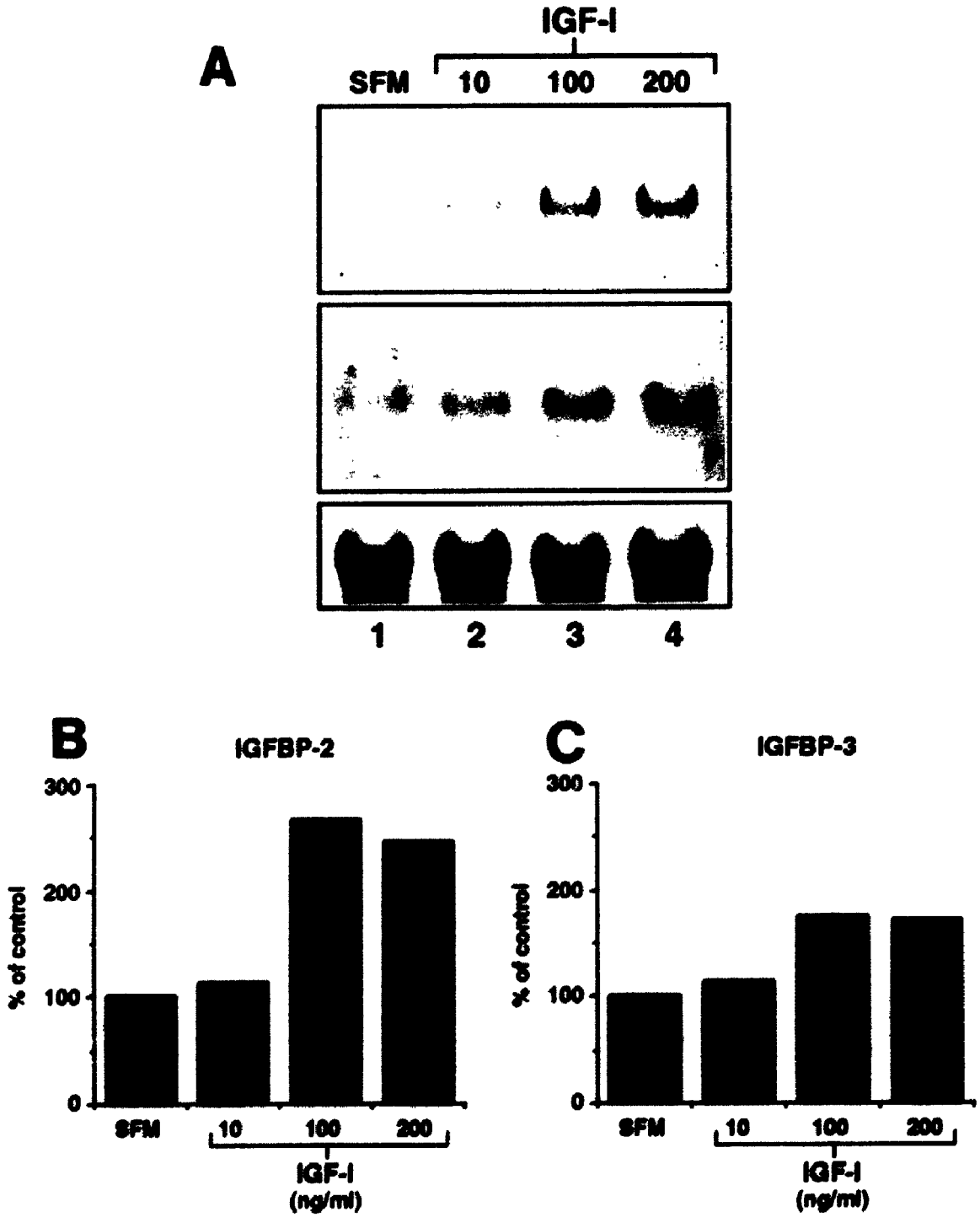
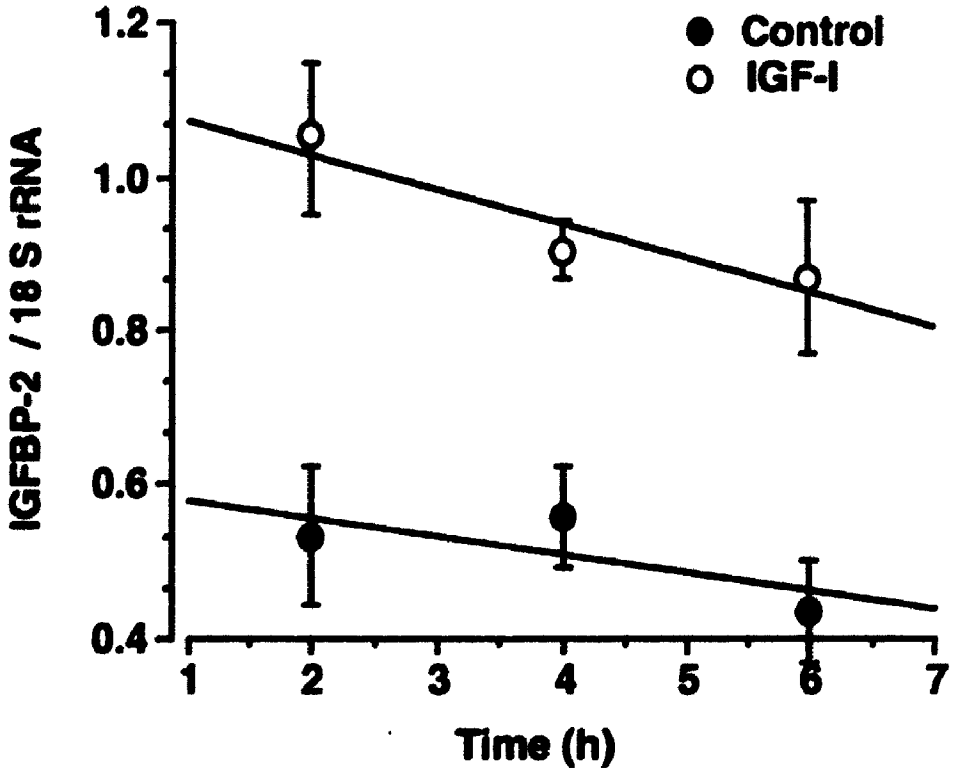

A
IGF-I
SFM
10
100
200
1
2
3
4
B
IGFBP-2
% of control
300
200
100
0
SFM
10
100
200
IGF-I
(ng/ml)
C
IGFBP-3
% of control
300
200
100
0
SFM
10
100
200
IGF-I
(ng/ml)

Figure 3.7. Astroglial were serum starved for 24 h, treated with or without IGF-I (200 ng/ml) for 24 h, actinomycin-D was added, the incubation was stopped at 2, 4 and 6 h, total RNA was extracted and IGFBP-2 mRNA levels were analyzed by slot blotting. The slot blot (triplicate samples) was quantified by densitometry and the mean ± SEM levels of IGFBP-2 mRNA were plotted against time.


Control
IGF-I
IGFBP-2 / 18 S rRNA
1.2
1.0
0.8
0.6
0.4
1
2
3
4
5
6
7
Time (h)

3.4.8 Cell specific expression of IGFBP mRNAs

In situ hybridization with specific antisense IGFBP-2 and IGFBP-3 cRNAs localized both IGFBP mRNAs to two populations of GFAP positive astroglial cells, the flat polygonal and smaller round process bearing cells (Figure 3.8). The specificity of hybridization was demonstrated by the lack of hybridization signal in cells treated with RNAse prior to hybridization (Figure 3.8 C, F). The radiolabeled IGFBP-2 sense RNA probe demonstrated a widespread hybridization which did not appear specific. It was subsequently shown to hybridize non-specifically with 28 S ribosomal RNA on a Northern blot (data not shown). Therefore, it was not useful as a control probe to determine specificity.

The cell density and the percentage of flat polygonal cells and round cells remained unchanged in all treatment groups indicating that IGF treatment for 24 h did not change the cell population (Table 3.1). The hybridization signal varied among cells indicating that at any one time, only a population of cells were expressing the mRNA. Treatment of astroglia with IGF-I resulted in an increase in the number of cells with hybridization signal for IGFBP-2 compared to cells maintained in basal conditions.

The mean number of grains per cell as an indicator of mRNA abundance per cell was increased by both IGF-I and IGF-II in flat polygonal cells (Table 3.2). Only IGF-II significantly increased the mean number of grains in flat polygonal astroglia hybridized with IGFBP-3 cRNA. The small round cells, present in lower numbers, responded to IGF-I and IGF-II by significantly increasing the number of grains per cell over control for both IGFBP-2 and IGFBP-3. The increase in IGFBP-2 stable mRNA levels upon IGF-I treatment reflected both an increase in the number of cells expressing the mRNA as well as increased mRNA levels in each cell resulting in a larger

Figure 3.8. Bright field photomicrographs of astroglial cultures analyzed for IGFBP-2 mRNA (**A, B** and **C**) and IGFBP-3 mRNA (**D, E** and **F**) by *in situ* hybridization using specific [^{35}S]IGFBP cRNA probes. Astroglia were treated with SFM (**A** and **D**) or IGF-I (200 ng/ml) (**B** and **E**). Large arrowheads indicate the flat, polygonal astroglia and the small arrowheads indicate the round, process bearing cells, both of which demonstrate GFAP immunoreactivity. Cultures hybridized following RNAse pretreatment or hybridized with a sense probe, have negligible hybridization for IGFBP-2 (**C**) or IGFBP-3 (**F**).

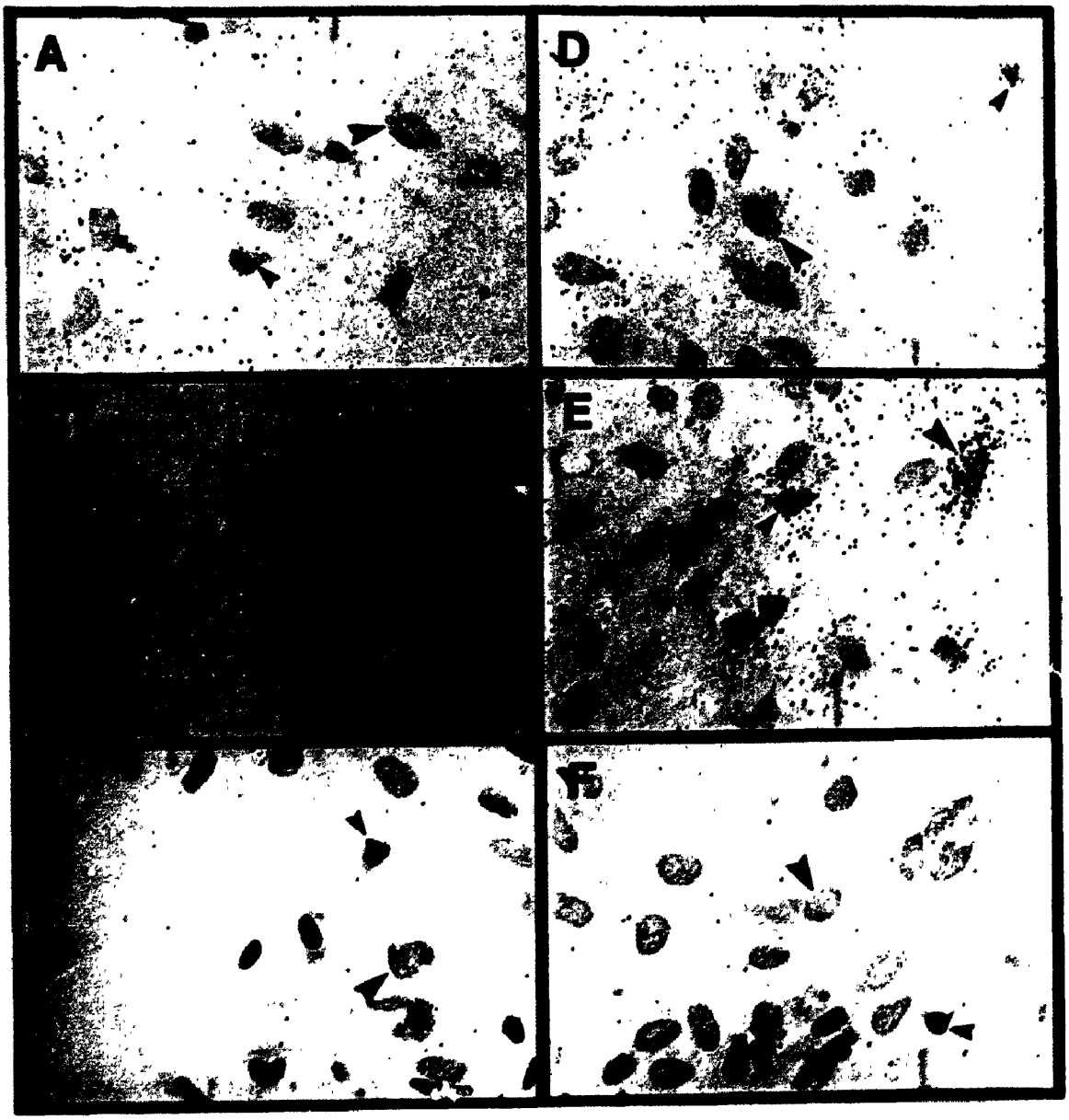

A
D
E
F

Table 3.1. Cell counts of the two cell types under control, IGF-I (200 ng/ml), or IGF-II (200 ng/ml) treatment conditions. The cell density for each treatment group was determined by counting the number of cells per field in thirty random fields and the mean number ± SEM of cells per field was determined from one experiment. The number of flat and round cells was counted in the same random fields and the mean percentage of each cell type was calculated. The cell density and the relative proportion of each cell type were not changed between different treatments indicating that the changes observed in IGFBP-2 or IGFBP-3 mRNA or protein levels were not due to alterations in cell number or type.

	Cell Density (cells / field)	Flat cells (%)	Round cells (%)
SFM	102 ± 24	91.2 ± 2.5	8.8 ± 2.5
IGF-I (200 ng/ml)	90 ± 20	89.6 ± 3.2	10.4 ±3.2
IGF-II (200 ng/ml)	98 ± 23	92.0 ± 2.7	8.0 ± 2.7

Table 3.2. The abundance of IGFBP-2 and IGFBP-3 mRNA in flat and round astroglia under control, IGF-I (200 ng/ml), or IGF-II (200 ng/ml) treatment conditions. The mean number of grains per cell, as a measure of IGFBP-2 or IGFBP-3 mRNA cellular abundance, was determined by counting the number of grains per cell in both flat and round cells in thirty random fields from a single experiment for all treatments. The mean and SEM were compared using Student's t-test. A statistically significant difference was determined when $p<0.05$ (*).

	Control	IGF-I	IGF-II
Flat			
IGFBP-2	8.5 ± 0.7	12.7 ± 0.9*	14.6 ± 1.1*
IGFBP-3	7.7 ± 0.5	7.0 ± 0.6	11.4 ± 0.6*
Round			
IGFBP-2	6.7 ± 0.7	20.7 ± 1.4*	9.0 ± 0.6*
IGFBP-3	8.1 ± 0.4	14.2 ± 0.6*	14.6 ± 0.6*

pool of stable mRNA. However, the increase in IGFBP-3 stable mRNA levels in response to IGF-I was observed only in the round cells which constituted only 10% of the whole population of glial cells. *In situ* hybridization, therefore, identified a cell specific response which may not have been observed by Northern analysis of total cellular RNA.

3.5 DISCUSSION

3.5.1 Characterization of astroglial IGFBPs

In the present study, we have demonstrated that primary astroglia synthesized two IGFBPs of 34 kDa and 40-45 kDa, which were shown to be IGFBP-2 and IGFBP-3 respectively, and that their expression was differentially regulated by IGFs and insulin. The 34 kDa IGFBP was shown previously to be IGFBP-2 by Western blotting using an antiserum against BRL-3A binding protein (Olson et al. 1991, Ocrant et al. 1990). The 40-45 kDa species could not be characterized immunologically as IGFBP-3 due to the unavailability of a specific antiserum. However, its apparent molecular size, its detection as a broad smear by ligand blotting, and the detection of a 2.4 kb transcript in Northern blots hybridized with a rat IGFBP-3 cDNA, indicated that this protein was most likely IGFBP-3.

IGFBP-2 is detected abundantly in the brain and CSF (Ocrant et al. 1990, Rosenfeld et al. 1989), and has a preferential affinity for IGF-II, the predominant IGF in the brain (Roghani et al. 1991a, 1991b). Postnatally, IGFBP-2 levels in the brain remain high while serum levels decline, suggesting that IGFBP-2 is an important modulator of IGF biological activity in the brain (Lamson et al. 1989). The 40-45 kDa IGFBP-3 has also been

detected in adult rat CSF, and conditioned media from neonatal rat astrocytes and C6 glioma cells (Han et al. 1988b, Ocrant et al. 1990). The present study demonstrates that IGFBP-2 and IGFBP-3 are produced by astroglial cells in culture.

3.5.2 Differential effects of IGF-I, IGF-II, and insulin on astroglial IGFBP levels

Both IGF-I and IGF-II increased the levels of IGFBP-2 and IGFBP-3 in astroglial conditioned media in a dose dependent manner, indicating that their effects were specific. This increase could not be accounted for by an increase in the synthesis of total secretory proteins, as the total protein content of the conditioned media remained relatively unchanged with IGF or insulin treatment. IGF-II appeared to be slightly more potent than IGF-I in stimulating IGFBP synthesis, achieving maximal stimulation at 100 ng/ml, whereas 200 ng/ml of IGF-I was required to achieve a similar effect. This difference between the two IGFs may be associated with the relative mitogenic potency of the two IGFs on astroglia. In the same cell type, it was previously shown that IGF-I is a more potent mitogen than IGF-II, with an ED_{50} of 2.0 ng/ml compared to 25 ng/ml respectively (Han et al. 1987b, Han et al. 1992).

We have observed previously that a significant [^{3}H]thymidine incorporation response of astroglia to IGF-II does not occur in concentrations less than 50 ng/ml and the maximum response is not achieved until 250 ng/ml. In addition, we have also demonstrated that IGFBP-2 inhibits the mitogenic actions of IGFs (Han et al. 1988b). These observations suggest that the initial blunted incorporation of [^{3}H]thymidine in response to IGF-II may be related to an increase in IGFBP-2 synthesis.

The presence of IGFBP-3 has been shown to be associated with both a reduction (DeMellow and Baxter 1988, Conover et al. 1990) as well as an enhancement (DeMellow and Baxter 1988, Blum et al. 1989) of IGF biological action. The biological effects of IGFBP-3 appears to be associated with its concentration relative to the IGFs and other IGFBPs, the temporal relationship to IGFs with regard to their presence in the extracellular *milieu,* and whether it is associated with the cell surface (Clemmons et al. 1991). Whether IGFBP-3 may be a positive or negative modulator of IGF action in astroglial cells is unknown.

Insulin reproduced the effects of IGFs at pharmacologic doses. Insulin stimulated a maximal increase in IGFBP-2 at 1 μg/ml and a maximal increased in IGFBP-3 at 10 μg/ml. Both types of IGF receptors as well as insulin receptors have been shown previously to be present in astroglia (Han et al. 1987b). The insulin effects may be mediated through the type 1 (IGF-I) receptor due to the very high concentrations used, although an interaction with the insulin receptor cannot be discounted.

The regulation of IGFBP-2 and/or IGFBP-3 synthesis by IGFs and insulin has been reported in a variety of cell types, and the degree and nature of response appears to be cell type dependent. In rat osteoblast-like cells, IGF-I is a potent stimulator of IGFBP-2 synthesis (Chen et al. 1991), whereas in cultured rat hepatocytes, IGFBP-2 gene expression is inhibited by insulin and pharmacologic concentrations of IGF-I (Boni-Schnetzler et al. 1990). In many different tissue systems *in vitro,* IGF-I increases IGFBP-3 synthesis (Smith et al. 1990, Conover 1990, Ceda et al. 1991, Neely and Rosenfeld 1992, Martin et al. 1992).

3.5.3 Differential effects of IGF-I, IGF-II, and insulin on IGFBP stable mRNA levels

The IGFBP-2 stable mRNA levels were increased in a dose dependent manner with 24 h of treatment by IGFs and insulin. This corresponded to the increase in IGFBP-2 protein levels in the conditioned medium detected by ligand blotting. In contrast, IGFBP-3 stable mRNA levels were unchanged with 24 h of treatment by IGFs or insulin, even though the IGFBP-3 protein levels were increased. The disparity between the IGFBP-3 mRNA and protein levels at 24 h suggests that either IGFBP-3 synthesis is regulated at a post-transcriptional level or that changes in the level of IGFBP-3 stable mRNAs are occuring at an earlier time point. To investigate the latter possibility, IGFBP-3 mRNA and protein levels were analyzed in the astroglial cells and conditioned media respectively, following treatment with IGF-I for different time periods. IGFBP-3 stable mRNA was increased rapidly to maximum levels within 12 h of treatment followed by a decline to control levels by 18 and 24 h, thus explaining why an increase in IGFBP-3 mRNA level was not observed at 24 h. However, IGFBP-3 stable mRNA levels were increased dose dependently by IGF-I when analyzed at 12 h, indicating that IGF-I has a rapid and specific effect on IGFBP-3 gene expression. This effect also appeared to be transient compared with the IGF-I effects on IGFBP-2 gene expression. Similar effects of IGF-I on IGFBP-3 gene expression have been observed in other cell types. Martin et al. (1992) demonstrated that in fibroblasts, stable IGFBP-3 mRNA levels were not different from control conditions after treatment with IGF-I for 48 h, even though IGFBP-3 protein levels in the conditioned media were elevated after 24 h. In bovine fibroblasts, Bale and Conover (1992) demonstrated that the IGF-I induced increase in IGFBP-3 stable mRNA levels reached maximum by 6 h, with a subsequent decline at

24 h. The release of cell surface associated IGFBP-3 by an IGF-I induced protease activity can also account for the apparent increase in IGFBP-3 secretion into the conditioned media of some cells (Conover 1992). Activity of an IGFBP-3 specific protease has not been observed in conditioned media from astroglia (Bradshaw et al. 1993).

The increase in IGFBP-2 stable mRNA at 24 h could reflect either an increase in transcription or an increase in stability of the mRNA. We have demonstrated that IGF-I did not alter the rate of decay of IGFBP-2 mRNA, suggesting that stability was not affected. It is possible that the concentration of actinomycin used did not effectively inhibit transcriptional activity, and therefore changes in stability would not have been accurately reflected. However, it is more likely that the increased mRNA levels were a result of increased gene transcription since the IGFBP-2 stable mRNA levels were induced rapidly. The rapid increase in IGFBP-2 mRNA levels could reflect induction by early immediate response elements. Several possible sites for recognition by transcription factors, such as AP-1, with the promoter region of rat IGFBP-2 have been described (Brown and Rechler 1990).

IGFBP-2 and IGFBP-3 mRNAs were localized to two populations of GFAP positive cells in the astroglial cultures. The flat polygonal cells, comprising the majority of the culture are protoplasmic astrocytes. The round, process bearing cells which comprised only 10% of the GFAP positive cells are likely fibrous astrocytes. Treatment with either IGF-I or -II resulted in a significant increase in the mean number of cells showing positive hybridization with IGFBP-2. This would suggest that IGFs increase the recruitment of cells expressing the IGFBP-2 gene. It was also evident that IGFs increased the intensity of hybridization per cell suggesting that there was increased mRNA levels in responsive cells. Positive hybridization for IGFBP-

3 was not significantly increased with IGF-I probably due to the transient gene induction observed with IGF-I. IGF-II treatment, however, resulted in a significant increase in hybridization signal for IGFBP-3 in contrast to our results from Northern analysis at 24 h and the time course of gene induction. This discrepancy can be explained by the fact that the *in situ* hybridization technique is more sensitive in detecting changes within single cells or a population of cells in comparison to Northern blotting which detects changes within a whole population of cells.

3.6 CONCLUSION

The regulation of IGFBPs by their endogenous ligands (IGFs) suggests the presence of an autocrine/paracrine loop mechanism for maintaining IGF and IGFBP homeostasis within this tissue system. The presence of increased IGF levels in the extracellular *milieu* would result in the increased activation of IGFBP genes and an increase in the production and secretion of IGFBPs. The increased levels of IGFBPs could sequester excess IGFs and thus modulate their biological activity upon the cells. The different bioactivities of IGF-I and IGF-II in regulating IGFBP biosynthesis could translate into differences in mitogenic activity. This feedback mechanism would be important in maintaining cellular homeostasis and regulated growth which is crucial to the developmental process. However, the exact function of the astroglial IGFBPs will need to be elucidated to determine their biological effect on growth of this cell type.

CHAPTER FOUR

REGULATION OF ASTROGLIAL IGFBP BIOSYNTHESIS BY GROWTH FACTORS PRODUCED LOCALLY IN THE BRAIN

4.1 INTRODUCTION

4.1.1 Epidermal growth factor

Epidermal growth factor (EGF) is a 53 amino acid, 6 kDa single-chain polypeptide that is synthesized as a 1200 amino acid (130 kDa) precursor (Carpenter and Wahl 1990). The sequence for mature EGF is located near the carboxyl terminal of the precursor molecule and is released by specific proteolytic cleavage. Human and mouse EGF share 70% amino acid homology (Gray et al. 1983, Bell et al. 1986). Expression of EGF mRNA is most abundant in the male mouse submaxillary gland and is also detected in the liver, kidney, lung, gastrointestinal tract and brain (Scott et al. 1983, Rall et al. 1985). EGF is a potent mitogen for many cell types of epithelial and mesenchymal origin and promotes the differentiation of numerous cell types (Carpenter and Wahl 1990).

4.1.2 Epidermal growth factor receptor

The rat epidermal growth factor receptor (EGFR) is a 170 kDa transmembrane glycoprotein that contains a high affinity binding site for EGF and EGF-like molecules (Ullrich et al. 1984). The EGF receptor can be subdivided into an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic domain with intrinsic tyrosine kinase activity. The extracellular ligand binding domain of the EGF receptor is characterized by a high content of cysteine residues that participate in disulfide bridges. The carboxyl terminal half of the receptor that contains the cytoplasmic tyrosine kinase domain has a high degree of homology with the v-*erb* B oncogene (Yamamoto et al. 1983, Ullrich et al. 1984).

4.1.3 Transforming growth factor-α

Transforming growth factor-α (TGF–α) is a polypeptide that shares 35% amino acid homology with EGF and binds with high affinity to the EGFR (Carpenter and Wahl 1990). The rat TGF-α gene encodes for a mRNA of 4.5 kb long. The mRNA is translated into a 160 amino acid trans-membrane glycoprotein precursor. Proteolytic cleavage, at specific sites, releases a soluble 50 amino acid extracellular domain which contains the biologically active region that is able to bind to the EGFR. The soluble TGF-α contains the characteristic "EGF unit", a sequence containing six spaced cysteines ($CX_7CX_{4-5}CX_{10-13}CXCX_8C$) which are linked in a defined configuration by three disulfide bridges (Derynck 1992).

The expression of TGF-α is most abundant in tumour cells and cells transformed by retroviruses however, it has also been detected in a variety of normal cell types. During development, TGF-α expression has been detected in pre-implantation blastocysts, many tissues in the mouse fetus and in the maternal decidua. TGF-α is a potent mitogen for cells of epithelial and mesenchymal origin and can induce cell migration, angiogenesis, and bone resorption (Carpenter and Wahl 1990, Derynck 1992).

4.1.4 Expression of EGF, TGF-α, and EGFR in the developing brain

The presence of EGF receptors in the developing and mature rodent brain has been deduced by the presence of EGFR mRNA (Kaser et al. 1992, Seroogy et al. 1995) and immunoreactive peptide (Fallon et al. 1984, Gomez-Pinilla et al. 1988). In particular, EGFR mRNA has been localized to germinal zones of the forebrain and cerebellum of the developing rat brain (Seroogy et al. 1995). EGFR immunoreactivity has been detected in astroglia of the

postnatal day 16 rat, peaking at day 19, and declining into adulthood (Gomez-Pinilla et al. 1988).

EGF and TGF-α mRNAs (Wilcox and Derynck 1988, Seroogy et al. 1991, Lazar et al. 1992) and immunoreactive peptides (Schaudies et al. 1989, Ferrer et al. 1995) have also been localized to widespread regions of the developing and adult rodent brain. EGF and TGF-α mRNAs were detected as early as embryonic day 14 and reached adult levels by postnatal day 10. The abundance of TGF-α mRNA was much higher than EGF in the developing and adult mouse brain (Lazar and Blum 1992).

The detection of EGF, TGF-α, and EGFR in the developing rodent brain suggest that EGF, TGF-α may play a role in the growth and development of the CNS. Studies *in vitro* and *in vivo* provide evidence for this hypothesis. EGF and TGF-α are potent mitogens for cultured neurons and astroglial cells (Honegger et al. 1983, Avola et al. 1988, Han et al. 1992, Chernausek 1993). The mitogenic activity of EGF on astroglial cells may be partly attributable to increased local synthesis of IGFs (Han et al. 1992, Chernausek 1993). EGF also acts as a neurotrophic, survival and maintenance factor for certain neuronal populations (Eccleston et al. 1985, Morrison et al. 1987). Mice that lack the EGFR gene have reduced brain size by embryonic day 18.5 and cytoarchitectural deformities suggesting abnormalities in cell migration and neuronal survival during development (Threadgill et al. 1995).

4.1.5 Fibroblast growth factors

Fibroblast growth factors (FGF) are a family of structurally related heparin-binding growth factors originally named because they promoted the growth of cultured fibroblasts (Baird and Bohlen 1990). To date, there are nine members of the FGF family, although acidic FGF (FGF-1) and basic FGF

(FGF-2) are the best characterized (Baird 1994). The biological actions of all members of the family remain to be elucidated although acidic and basic FGF have potent mitogenic and angiogenic activities (Baird and Bohlen 1990, Basilico and Moscatelli 1992).

Basic FGF is a single chain polypeptide of 146 amino acids with a molecular weight of 16.5 kDa, although longer variants have been described (Esch et al. 1985, Baird 1994). Acidic FGF is 140 amino acid protein with a molecular weight of 15.5 kDa. Basic and acidic FGF share about 55% sequence homology and both lack a signal peptide sequence. The mechanism by which acidic and basic FGF are secreted outside the cell remains unknown.

4.1.6 Fibroblast growth factor receptors

A family of high affinity FGF receptors (FGFR) have been described that are tyrosine kinase transmembrane proteins and members of the immunoglobulin IgG superfamily (Johnson and Williams 1993). Four members of the FGF receptor gene family have been identified. The FGFR, in their largest form, consist of three extracellular IgG domains, an acid box domain, a transmembrane region, and a cytoplasmic tyrosine kinase domain. Structurally variant isoforms of each receptor have been characterized. The variant isoforms have deletions in the first IgG domain, alternative exon usage in the third IgG domain, and alternative stop codon usage generating secreted truncated forms of FGFR1 lacking the transmembrane and cytoplasmic domains (Lee et al. 1989, Hou et al. 1991, Dell and Williams 1992, Johnson and Williams 1993). All of the FGFR bind both acidic FGF and basic FGF except for FGFR4 which does not bind basic FGF. FGFs also bind to cell surface heparan sulfate proteoglycans that act as low affinity receptors (Kiefer

et al. 1990). Binding of FGF to the low affinity receptor enables the interaction of FGF with its high affinity receptors (Ornitz et al. 1992, Rapraeger et al. 1991).

4.1.7 Expression of acidic FGF, basic FGF, and FGF receptors in the developing brain

During development of the rat brain, acidic FGF, basic FGF and their receptors have distinct spatio-temporal localization in both glial cells and neurons suggesting that they have an important role in the processes of cell proliferation, migration, and axon sprouting (Wilcox and Unnerstall 1991, Stock et al. 1992, El-Husseini et al. 1994, Gomez-Pinilla et al. 1994). Acidic FGF mRNA is detectable as early as embryonic day 11 in the murine CNS, at embryonic day 18 is associated with the developing cortical plate, and is expressed into adulthood (Wilcox and Unnerstall 1991, Nurcombe et al. 1993). Acidic FGF mRNA expression has been localized predominantly to neurons, and correlates with the differentiation and migration of neurons in the hippocampus, cerebellum, and specific cortical areas (Wilcox and Unnerstall 1991). Basic FGF can be detected as early as embryonic day 9 associated with the primitive cerebral cortex (Nurcombe et al. 1993). Postnatally, basic FGF is associated with astrocytes throughout the brain and in specific neuronal populations of the hippocampus and cingulate cortex (Ernfors et al. 1990, Powell et al. 1991, Gomez-Pinalla et al. 1994). FGFR1 and FGFR2 mRNAs have been detected throughout the postnatal rat brain with regional variation (El-Husseini et al. 1994). In general, FGFR1 is expressed in neuronal cells and FGFR2 is expressed in astroglial cells (Johnson and Williams 1993).

Studies *in vitro* have demonstrated that basic FGF is a potent mitogen for astroglial cells (Torres-Aleman et al. 1990, Engele and Bohn 1992) and can stimulate their differentiation (Lillien and Raff 1990). Both FGFs have potent

neurotrophic activities in that they induce the proliferation, differentiation, and enhance the survival of neurons in culture (Walicke et al. 1986, Walicke 1988, Torres-Aleman et al. 1990, Ishikawa et al. 1992, Baird 1994).

4.2 OBJECTIVE

EGF, TGF-α, basic FGF and acidic FGF and their respective receptors have distinct spatio-temporal patterns of expression in the developing rat brain and these growth factors have been shown to stimulate the proliferation of astroglia in culture (Han et al. 1992, Chernausek 1993, Torres-Aleman et al. 1990, Engele and Bohn 1992). In addition, we have demonstrated that EGF can potentiate IGF-I stimulated [^{3}H]thymidine incorporation and increase the amount of radioimmunoassayable IGF-I produced by astroglia suggesting that a part of the biologic effect of these growth factors in the brain is mediated via the IGF system (Han et al. 1992). We hypothesized that these growth factors regulate the expression of IGFBPs, one compartment of the IGF system, in an autocrine/paracrine manner. As a first step in defining the interactions of these specific growth factors with the IGF system, the objective of this study was to determine whether EGF, TGF-α, basic FGF and acidic FGF regulate the expression of astroglial IGFBPs.

4.3 METHODS

4.3.1 Analysis of secreted IGFBPs

Conditioned media were collected from confluent cultures in T-75 flasks following 24 h incubation with either EGF, TGF-α, acidic FGF, or basic FGF (1, 10, 25 ng/ml). These growth factor concentrations were chosen based on previous experiments with EGF (Han et al. 1992) and basic FGF (unpublished data) that demonstrated a biologic response in astroglia as determined by thymidine incorporation. Heparin (70 μg/ml) was included in the culture media for incubation with both acidic FGF and basic FGF. Ligand blot analysis was performed as described in section 2.3 using [^{125}I]IGF-II as a radiolabel. For the time course studies, conditioned media were collected at the various time intervals indicated. Autoradiograms were quantified by laser densitometry (Abaton Scan300/Colour) and the Scan Analysis 2 program. Analysis of variance was used to determine statistically significant changes in IGFBP protein levels.

4.3.2 Analysis of IGFBP stable mRNA levels

Confluent cells in T-75 flasks were used for extraction of total RNA and subjected to Northern blot analysis as described in section 2.5. Changes in IGFBP stable mRNA levels were quantified by laser densitometry of the Northern blots autoradiograms (Personal Densitometer^{TM}, Model 375A, Molecular Dynamics Inc.). The relative densities of the bands were expressed as arbitrary absorbance units (au). To correct for minor differences in loading of total RNA, a ratio of the relative density of each specific band with the relative density of the 18S ribosomal band was calculated before comparisons were made.

The time course changes in IGFBP stable mRNA were expressed as a percentage of cells maintained in SFM for 24 h.

4.4 RESULTS

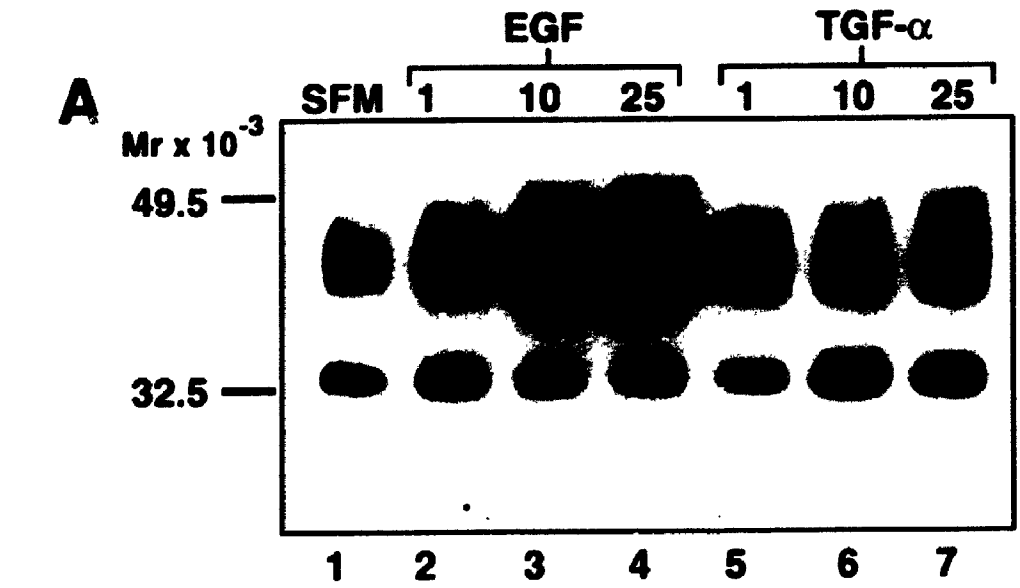
4.4.1 Exogenous EGF and TGF-α increase the levels of IGFBPs in astroglial conditioned media

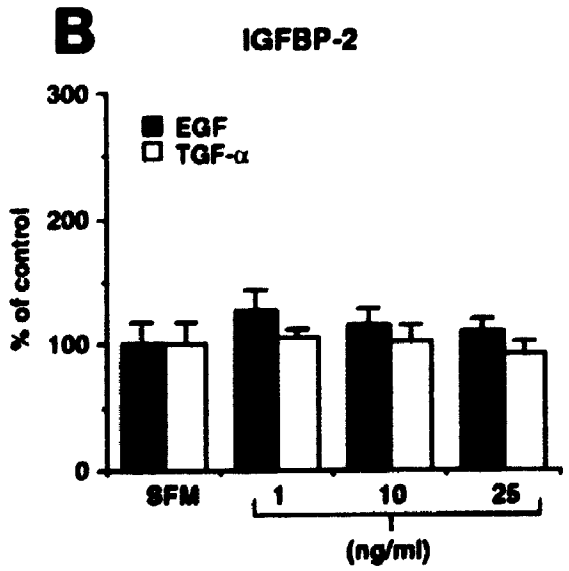
A representative ligand blot of conditioned media obtained from astroglial cultures treated with EGF (1, 10, 25 ng/ml) and TGF-α (1, 10, 25 ng/ml) for 24 h is shown in Figure 4.1 A. IGFBP-2 protein levels were increased slightly by EGF and TGF-α. Densitometric analysis of three separate experiments confirmed that EGF and TGF-α did not significantly affect IGFBP-2 protein levels however, EGF at the lowest dose tested (1 ng/ml) slightly increased IGFBP-2 levels to 127% of control levels (Figure 4.1 B). In contrast, both EGF and TGF-α increased the levels of IGFBP-3 in the conditioned media. Densitometric analysis of three separate experiments confirmed that EGF increased IGFBP-3 levels in a dose dependent manner. EGF, at the highest dose tested (25 ng/ml), stimulated a greater than 2-fold increase in IGFBP-3 protein levels compared to control conditions (Figure 4.1 C). TGF-α stimulated a maximal 1.7-fold increase in IGFBP-3 compared to control conditions (Figure 4.1 C). EGF was more potent than TGF-α in increasing the levels of IGFBP-3 detected in the conditioned media.

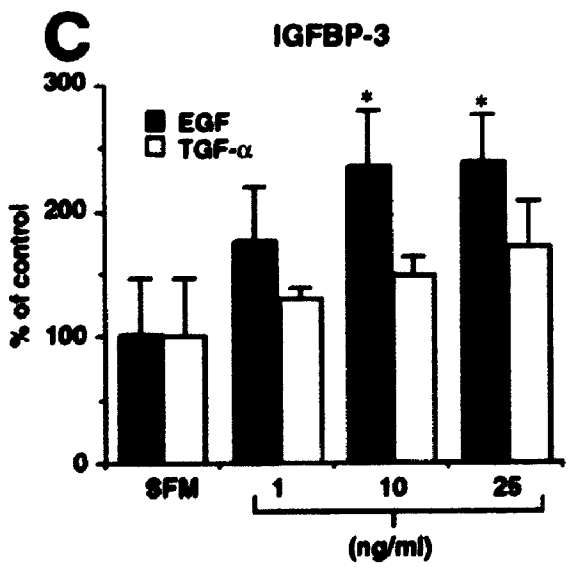
4.4.2 The effect of EGF and TGF-α on IGFBP stable mRNA levels

To determine whether the increased detection of IGFBP protein in the conditioned medium was due to an increase in stable mRNA levels, Northern blot analysis was performed on astroglial cultures treated for 24 h

Figure 4.1 Ligand blot analysis of conditioned media (CM) from astroglia treated with EGF and TGF-α. Conditioned media were collected from primary astroglia treated for 24 h with EGF or TGF-α, subjected to SDS-PAGE, transferred to nitrocellulose and incubated with [^{125}I]IGF-II. (**A**) Autoradiograph of a representative ligand blot showing, lane 1: SFM control, lanes 2-4: EGF (1, 10 and 25 ng/ml), and lanes 5-7: TGF-α (1, 10 and 25 ng/ml). (**B**) The densitometric analysis (three separate experiments) of changes in IGFBP-2 levels in the CM from astroglia treated with either EGF or TGF-α expressed as mean ± SEM of percent change from control. (C) A similar analysis for IGFBP-3. * indicates a statistically significant change ($p < 0.05$).


A
EGF
TGF-α
SFM
1
10
25
1
10
25
Mr x 10-3
49.5
32.5
1
2
3
4
5
6
7


B
IGFBP-2
EGF
TGF-α
% of control
300
200
100
0
SFM
1
10
25
(ng/ml)


C
IGFBP-3
EGF
TGF-α
% of control
300
200
100
0
*
*
SFM
1
10
25
(ng/ml)

with the same doses of EGF and TGF-α. Figure 4.2 A is a representative Northern blot of astroglial total RNA probed sequentially with radiolabeled IGFBP-2 and IGFBP-3 cDNAs. Transcripts of 1.6 kb and 2.6 kb were observed upon hybridization with IGFBP-2 and IGFBP-3 cDNAs respectively. Densitometric analysis of this Northern determined that stable IGFBP-2 mRNA levels were barely altered by TGF-α (Figure 4.2 B). EGF, however, stimulated a 2-fold increase in IGFBP-2 stable mRNA at the lowest dose tested (1 ng/ml) suggesting that the increased amount of IGFBP-2 in the conditioned media detected at this dose may be partly due to increased levels of stable mRNA. Stable IGFBP-3 mRNA levels were increased over 5-fold by EGF at 10 and 25 ng/ml (Figure 4.2 C). In contrast, TGF-α increased IGFBP-3 stable mRNA levels 1.5-fold of control levels. Similar trends were observed in a second experiment.

4.4.3 Time course of induction of IGFBP mRNA by EGF and TGF-α

The EGF and TGF-α induced changes in IGFBP stable mRNA were analyzed by Northern blotting, quantified by densitometry and expressed as a percentage of cells maintained in SFM for 24 h. The results for one experiment are shown in Figure 4.3. EGF induced a maximum response in IGFBP-2 stable mRNA levels by 6 h, which subsequently declined by 12 h and increased by 24 h to above control levels (Figure 4.3 A). TGF-α induced an increase in IGFBP-2 stable mRNA levels over time which reached a maximum at 24 h that did not exceed control levels. Stable IGFBP-3 mRNA levels increased over time after the addition of EGF reaching a maximum by 24 h (Figure 4.3 B). TGF-α treatment caused IGFBP-3 stable mRNA levels to increase over time reaching maximum levels by 24 h but did not exceed control levels. The discrepancy between the TGF-α induced changes in

Figure 4.2 (**A**) A representative Northern blot of total RNA prepared from astroglia treated with EGF or TGF-α, and probed sequentially with [^{32}P]labeled rat IGFBP-2 (upper panel), IGFBP-3 (middle panel), or 18 S ribosomal (lower panel) cDNAs. Lane 1: SFM control, lanes 2-4: EGF (1, 10, 25 ng/ml) and lanes 5-7: TGF-α (1, 10, 25 ng/ml). The changes in stable mRNA levels were quantified by densitometry of the Northern and expressed as the percent change from control for IGFBP-2 (**B**) and IGFBP-3 (**C**).

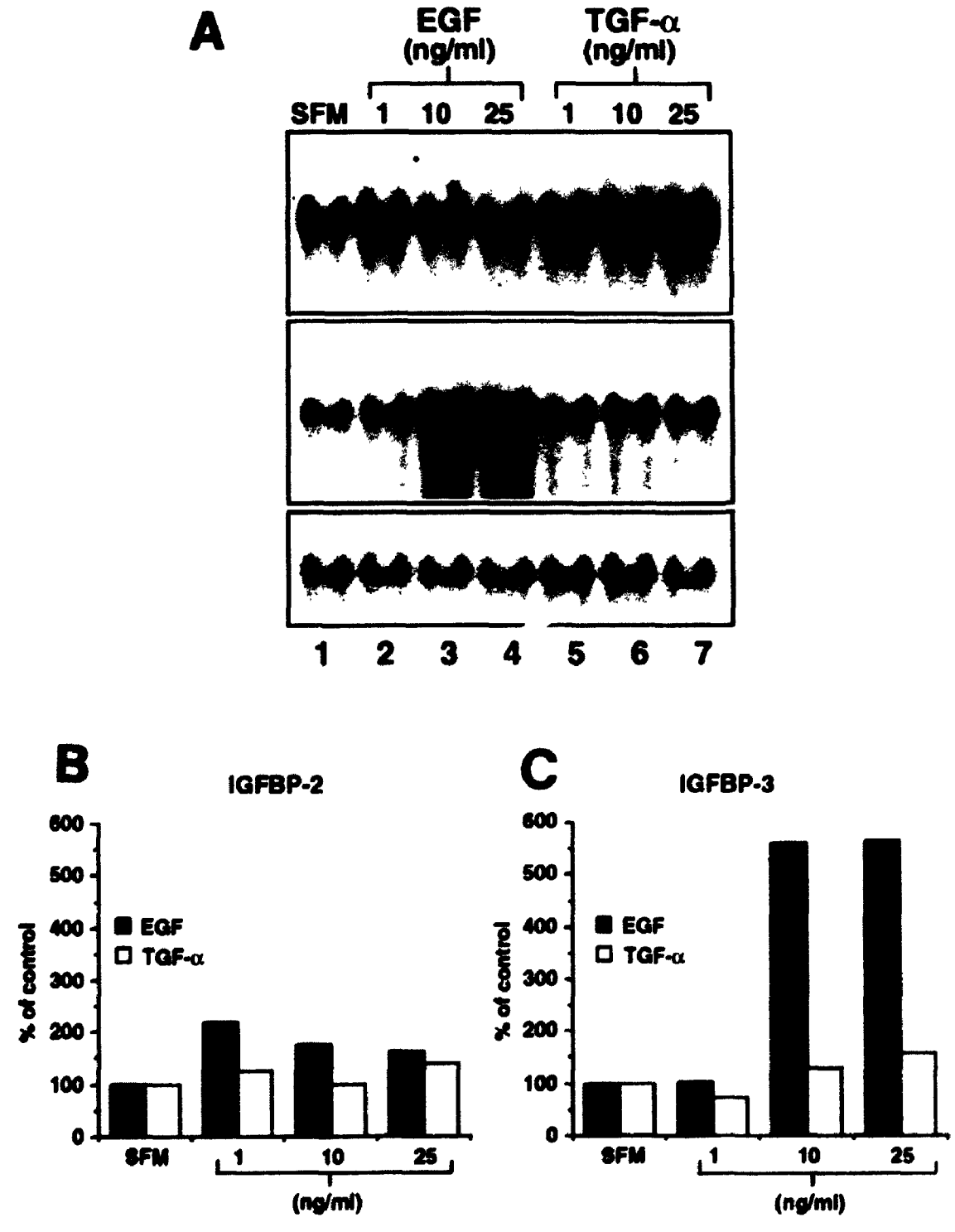
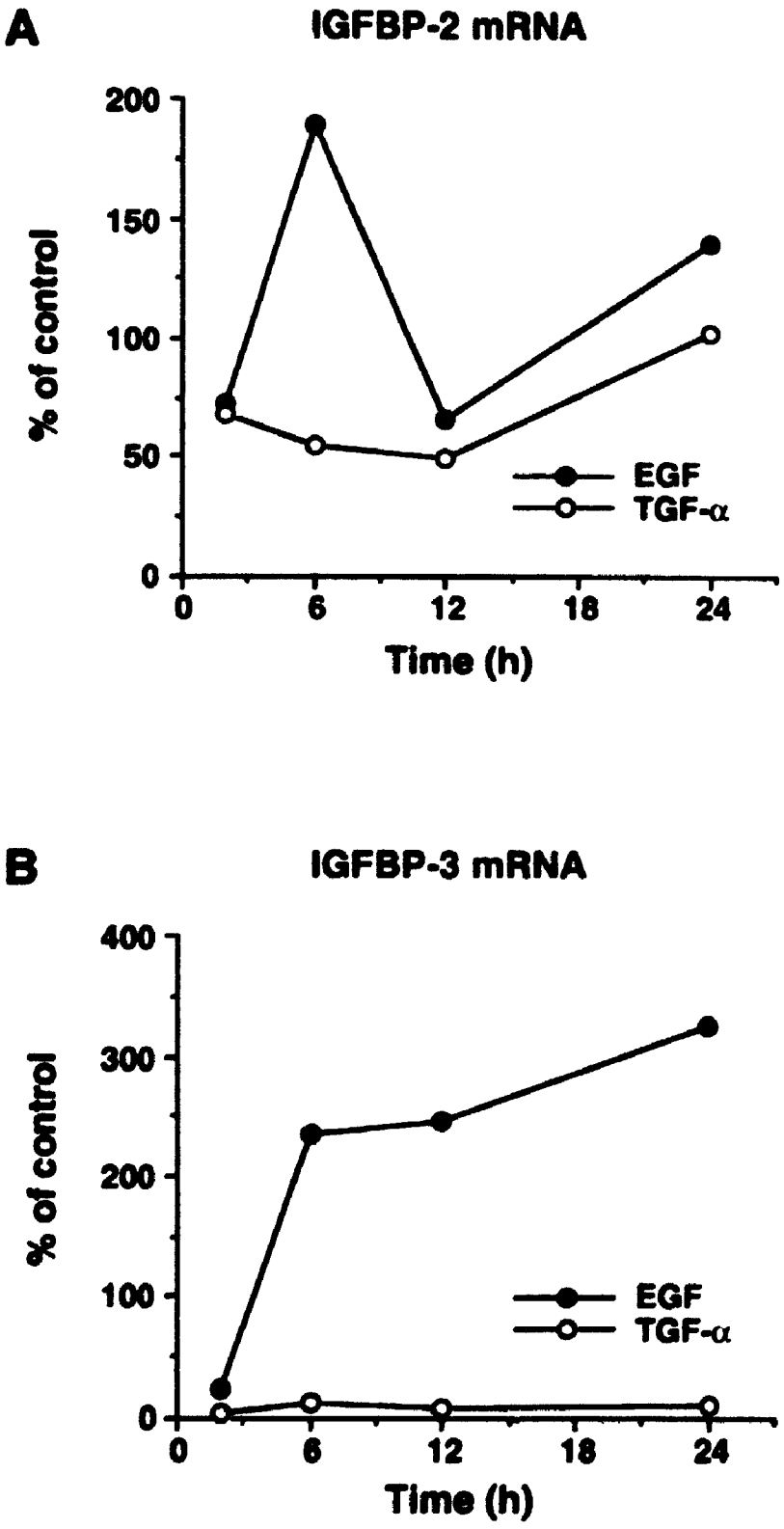

A
EGF (ng/ml)
TGF-α (ng/ml)
SFM 1 10 25 1 10 25
1 2 3 4 5 6 7
B
IGFBP-2
% of control
600
500
400
300
200
100
0
EGF
TGF-α
SFM 1 10 25
(ng/ml)
C
IGFBP-3
% of control
600
500
400
300
200
100
0
EGF
TGF-α
SFM 1 10 25
(ng/ml)

Figure 4.3 The time course of EGF and TGF-α induced expression of IGFBP-2 mRNA (**A**) and IGFBP-3 mRNA (**B**). Primary astroglia were treated with 25 ng/ml of EGF or TGF-α, and total RNA was isolated at various time points (2, 6, 12, and 24 h). Total RNA was analyzed by Northern blotting and changes in mRNA were quantified by densitometry and expressed as the percent change from control (cells maintained in SFM for 24 h). Similar results were observed in a second experiment.


A
IGFBP-2 mRNA
% of control
200
150
100
50
0
0
6
12
18
24
Time (h)
EGF
TGF-α
B
IGFBP-3 mRNA
% of control
400
300
200
100
0
0
6
12
18
24
Time (h)
EGF
TGF-α

IGFBP-3 stable mRNA at 24 h compared to the time course changes may be reflected by differences between separate cultures. Additional experiments confirmed the time course changes in IGFBP-2 and IGFBP-3 in response to EGF, however, the time course changes in response to TGF-α have been conflicting.

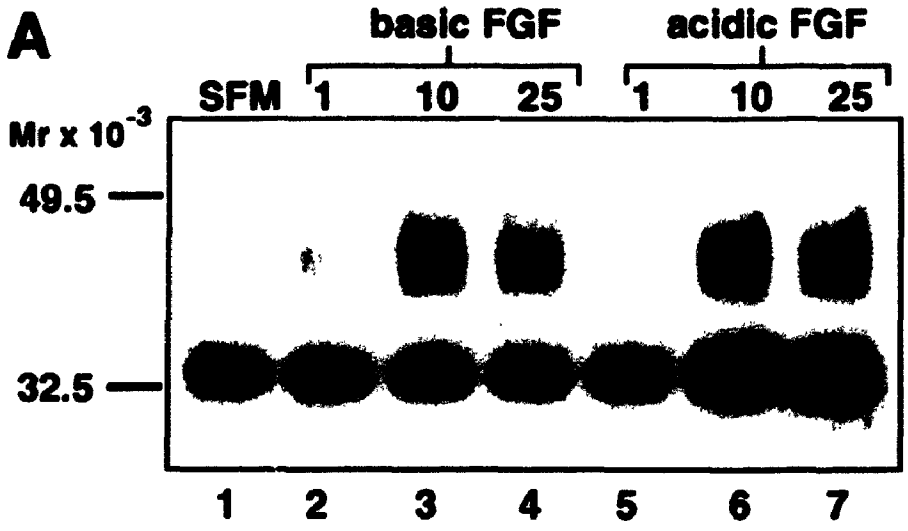
4.4.4 Exogenous acidic FGF and basic FGF increase astroglial IGFBPs

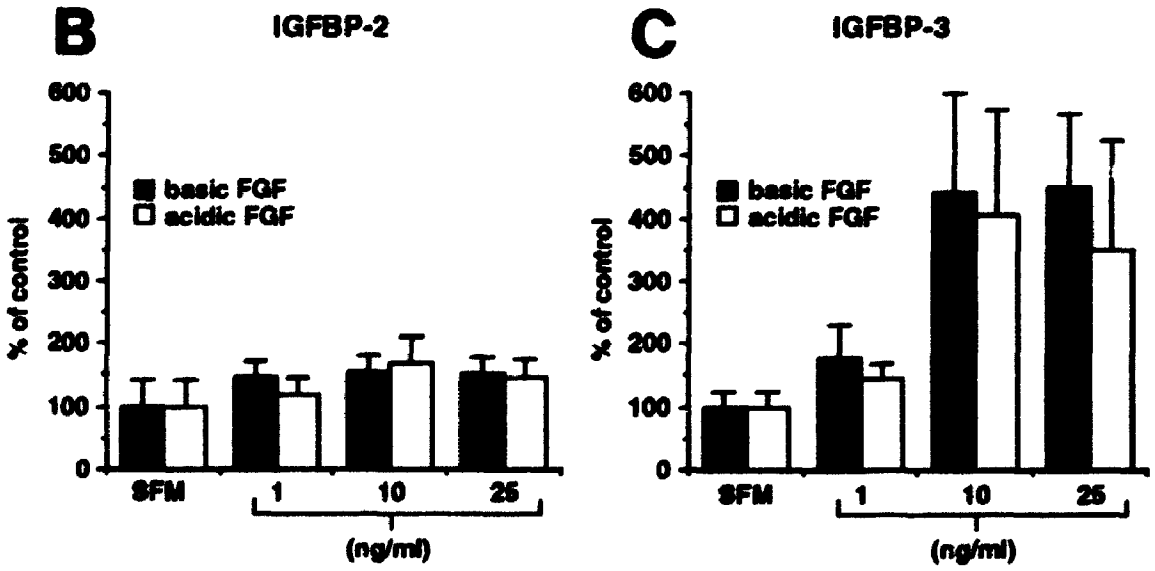
A representative ligand blot of conditioned media obtained from astroglial cultures treated with acidic FGF (1, 10, 25 ng/ml) and basic FGF (1, 10, 25 ng/ml) is shown in Figure 4.4 A. IGFBP-2 protein levels in the conditioned media were increased by acidic FGF and slightly increased by basic FGF. Densitometric analysis of three separate experiments demonstrated that acidic FGF and basic FGF increased IGFBP-2 protein levels to a maximum of 168% and 155% respectively compared to control levels (Figure 4.4 B). Both acidic FGF and basic FGF increased the levels of IGFBP-3 in the conditioned media. Densitometric analysis showed that acidic FGF increased IGFBP-3 protein levels in a dose dependent manner and stimulated a maximal 4-fold increase at a concentration of 10 ng/ml. Basic FGF stimulated a maximal 4.5-fold increase in IGFBP-3 protein levels compared to control conditions at the highest concentration used (25 ng/ml) (Figure 4.4 C).

4.4.5 The effect of acidic FGF and basic FGF on IGFBP stable mRNA levels

To determine whether the increase in IGFBP-2 and IGFBP-3 detected in the conditioned media were due to an increase in stable mRNA levels, Northern blot analysis was performed on astroglial cultures treated for 24 h with various doses of acidic FGF and basic FGF. Figure 4.5 A is a representative Northern blot of astroglial total RNA probed sequentially with

Figure 4.4 Ligand blot analysis of conditioned media (CM) from astroglia treated with basic FGF and acidic FGF. Conditioned media were collected from primary astroglia treated for 24 h with basic FGF or acidic FGF, subjected to SDS-PAGE, transferred to nitrocellulose and incubated with [^{125}I]IGF-II. **(A)** Autoradiograph of a representative ligand blot showing, lane 1: SFM control, lanes 2-4: basic FGF (1, 10 and 25 ng/ml), and lanes 5-7: acidic FGF (1, 10 and 25 ng/ml). **(B)** The densitometric analysis (three separate experiments) of changes in IGFBP-2 levels in the CM from astroglia treated with either basic FGF or acidic FGF expressed as mean $\pm$ SEM of percent change from control. **(C)** A similar analysis for IGFBP-3. No statistically significant changes were observed.


A
basic FGF
acidic FGF
SFM
1
10
25
1
10
25
Mr x 10-3
49.5
32.5
1
2
3
4
5
6
7

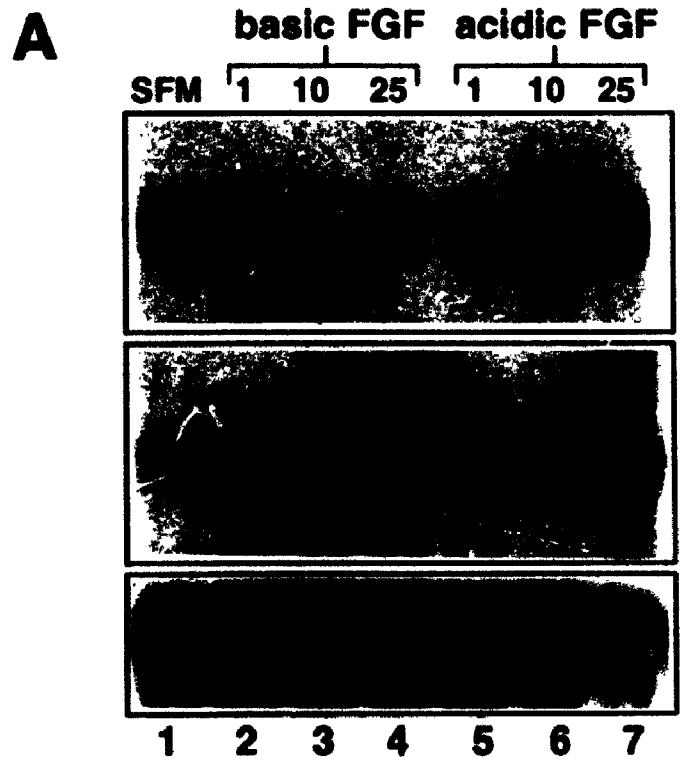

B
IGFBP-2
C
IGFBP-3
% of control
basic FGF
acidic FGF
SFM
1
10
25
(ng/ml)

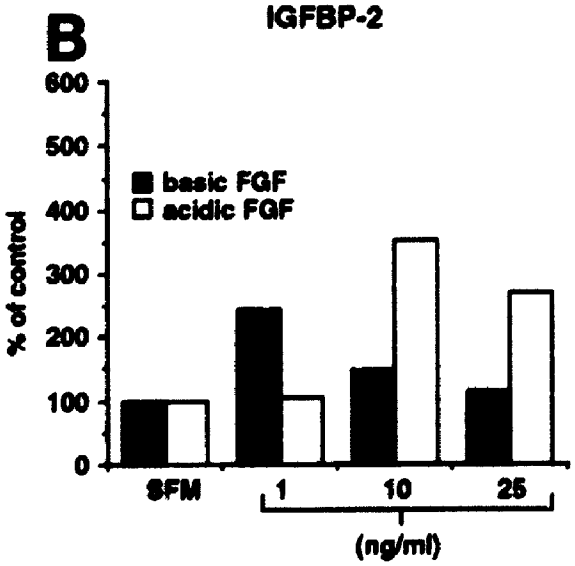
radiolabeled IGFBP-2 and IGFBP-3 cDNAs. Transcripts of 1.6 kb and 2.6 kb were observed upon hybridization with IGFBP-2 and IGFBP-3 cDNAs respectively. Densitometric analysis of the Northern blot showed that IGFBP-2 stable mRNA levels were increased over 2-fold by basic FGF at the lowest dose tested (1 ng/ml) (Figure 4.5 B). Acidic FGF increased stable IGFBP-2 mRNA levels over 3-fold at a concentration of 10 ng/ml. Stable IGFBP-3 mRNA levels were also increased by acidic FGF and basic FGF in a dose dependent manner. Basic FGF increased IGFBP-3 stable mRNA levels over 5-fold at the higher concentrations tested (10, 25 ng/ml) (Figure 4.5 C). Acidic FGF increased stable IGFBP-3 mRNA levels 3-fold at the highest concentration tested (25 ng/ml). In other experiments, a consistent response in IGFBP-2 and IGFBP-3 stable mRNA with basic FGF was observed however, the response to acidic FGF was variable.

4.4.6 Time course of induction of IGFBP stable mRNAs by acidic FGF and basic FGF

IGFBP-2 mRNA levels reached a maximum by 6 h upon treatment with basic FGF and declined thereafter to control levels at 24 h (Figure 4.6 A). Acidic FGF also maximally increased IGFBP-2 stable mRNA levels at 6 h. IGFBP-3 mRNA levels increased rapidly after the addition of basic FGF, reaching a maximum by 6 h, declined slightly thereafter, but remained above control levels at 24 h (Figure 4.6 B). Acidic FGF also rapidly induced a maximum increase in IGFBP-3 stable mRNA levels by 2 h which decreased thereafter but remained above control levels. The time course response of IGFBP-3 stable mRNA to acidic FGF and basic FGF was confirmed in other experiments however, the time course response of IGFBP-2 stable mRNA in response to acidic FGF was variable.

Figure 4.5 (**A**) A representative Northern blot of total RNA prepared from astroglia treated for 24 h with basic FGF or acidic FGF, and probed sequentially with [^{32}P]labeled rat IGFBP-2 (upper panel), IGFBP-3 (middle panel), and 18 S ribosomal (lower panel) cDNAs. Lane 1: SFM control, lanes 2-4: basic FGF (1, 10, 25 ng/ml) and lanes 5-7: acidic FGF (1, 10, 25 ng/ml). The changes in stable mRNA levels were quantified by densitometry and expressed as the percent change from control (cells maintained in SFM for 24 h) for IGFBP-2 (**B**) and IGFBP-3 (**C**).


A
basic FGF
acidic FGF
SFM
1
10
25
1
10
25
1 2 3 4 5 6 7


B
IGFBP-2
% of control
600
500
400
300
200
100
0
basic FGF
acidic FGF
SFM
1
10
25
(ng/ml)

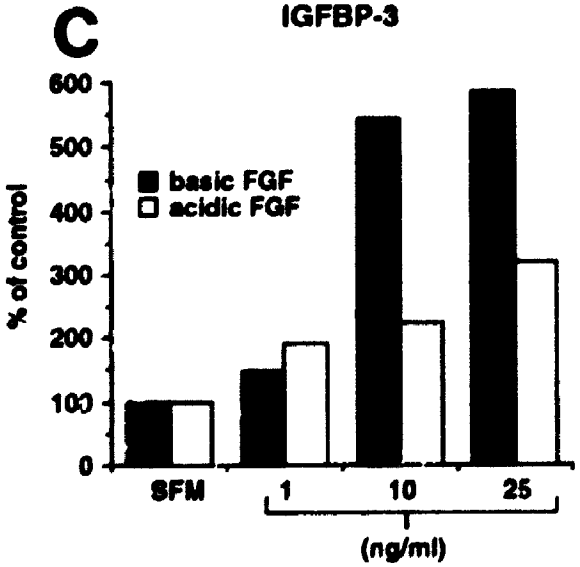
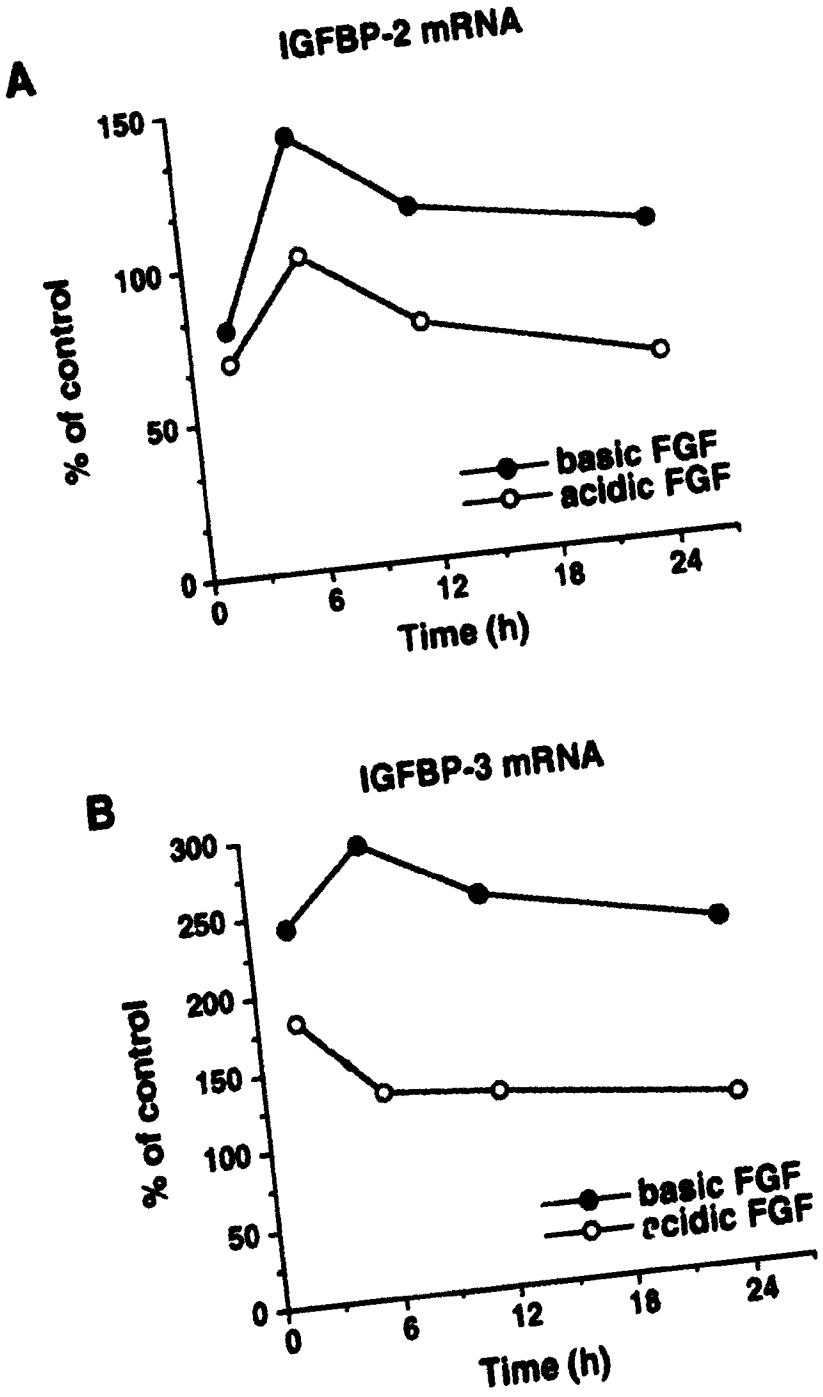

C
IGFBP-3
% of control
600
500
400
300
200
100
0
basic FGF
acidic FGF
SFM
1
10
25
(ng/ml)

Figure 4.6 The time course of basic FGF and acidic FGF induced expression of IGFBP-2 mRNA (**A**) and IGFBP-3 mRNA (**B**). Primary astroglia were treated with 25 ng/ml of basic FGF or acidic FGF, and total RNA was isolated at various time points (2, 6, 12, and 24 h). Total RNA was analyzed by Northern blotting and the changes in IGFBP mRNA were quantified by densitometry and expressed as the percent change from control (cells maintained in SFM for 24 h).


A
IGFBP-2 mRNA
% of control
150
100
50
0
0
6
12
18
24
Time (h)
basic FGF
acidic FGF
B
IGFBP-3 mRNA
% of control
300
250
200
150
100
50
0
0
6
12
18
24
Time (h)
basic FGF
acidic FGF

4.5 DISCUSSION

4.5.1 EGF and TGF-α differentially regulate the level of astroglial IGFBP-2 and IGFBP-3

The growth factors EGF and TGF-α differentially regulated the levels of astroglial IGFBP-2 and IGFBP-3 detected in the conditioned media. The amount of IGFBP-2 in the conditioned media was modestly increased by treatment with EGF for 24 h and had a maximum effect at the lowest dose tested. EGF increased IGFBP-2 stable mRNA levels at the lowest dose tested suggesting that the increased levels of IGFBP-2 detected by ligand blotting was due to increased levels of stable mRNA. The EGF induced increase in IGFBP-2 stable mRNA levels, which peaked at 6 h, suggested that EGF stimulates IGFBP-2 gene transcription. In contrast, the amount of IGFBP-2 in the conditioned media was relatively unaffected by TGF-α and IGFBP-2 stable mRNA levels were not increased by TGF-α. IGFBP-3 production was increased dramatically by both EGF and TGF-α in a dose dependent manner with EGF being more potent than TGF-α. The dramatic increase in IGFBP-3 stable mRNA levels by EGF suggested that the increased levels of IGFBP-3 in the conditioned medium was occurring partly at the transcriptional level. Similar to IGFBP-2, a rapid increase of IGFBP-3 stable mRNA levels was observed at 6 h. In contrast to IGFBP-2, the maximum increase was observed at 24 h. The effect of TGF-α on stable IGFBP-3 mRNA levels was much less compared to the effect of EGF suggesting that EGF and TGF-α are stimulating an increase in IGFBP-3 protein by different mechanisms. The difference in response of IGFBPs to EGF and TGF-α suggest that their effects are not due to a general increase in cellular protein synthesis, and binding of either of the two ligands to the EGFR does not lead to a similar change in IGFBP synthesis.

These results indicated that EGF and TGF-α have differential effects on stable IGFBP mRNA levels for IGFBP-2 and IGFBP-3. The increased stable mRNA levels can be attributed to either an increase in the rate of transcription or an increase in the stability of the mRNA. EGF and TGF-α may differentially affect transcription through differential second messenger systems. These results also indicated that EGF had differential effects on the regulation of IGFBP-2 and IGFBP-3. The characterized promoter regions of IGFBP-2 and IGFBP-3 have different consensus elements for the interaction of various transcription factors. In particular, the IGFBP-2 promoter does not contain a TATA box in contrast to the IGFBP-3 promoter (Brown and Rechler 1980, Albiston et al. 1995). Whether these differences in regulatory elements may account for the differential effects of EGF on IGFBP-2 and IGFBP-3 stable mRNA levels is unknown.

The increased levels of IGFBP detected in the conditioned media may also be due to post-transcriptional effects such as increased translation, increased rate of secretion of IGFBPs, or release of cell surface associated IGFBPs. Cell surface association has been described for both IGFBP-2 and IGFBP-3 in other cell systems (Conover et al. 1990, Oh et al. 1993a, 1993b, Reeve et al. 1993). This possibility was not examined in the current study, however, previous analysis of cell surface binding sites for [^{125}I]IGF-I or [^{125}I]IGF-II on primary astroglia failed to detect possible cell surface associated IGFBPs (Han et al. 1987). Alternatively, the increased levels of IGFBP protein detected in the conditioned media could be due to decreased degradation, however, IGFBP specific proteases have not been detected in the conditioned media of primary astroglia (see Chapter 5).

4.5.2 Possible mechanisms for the differential effects of EGF and TGF-α

EGF and TGF-α have different effects on the regulation of astroglial IGFBP-2 and IGFBP-3 both at the protein level and the stable mRNA level. EGF and TGF-α bind to the same transmembrane receptor, the EGFR, through which they exert their biologic effects. In certain assays, such as the stimulation of DNA synthesis, EGF and TGF-α are equally potent, however, in other assays, EGF and TGF-α have different potencies suggesting that they interact differently with the EGFR (Derynck 1992). Binding studies have determined that EGF and TGF-α have similar dissociation constants for the EGFR, however, TGF-α dissociates from the EGFR at a higher pH than EGF presumably due to its higher pI compared to EGF (Ebner and Derynck 1991). These studies suggest that there may be differences between EGF and TGF-α in receptor-ligand internalization, receptor-ligand recycling and receptor down-regulation, and differential activation of second messenger signaling systems.

An alternative mechanism for the different potencies of EGF and TGF-α may be due to their differential stability *in vitro*. Studies have suggested that EGF and TGF-α are degraded by separate pathways (Korc et al. 1987, Korc and Finman 1989, Garcia et al. 1989, Gehm and Rosner 1991). Korc and Finman (1989) have demonstrated that TGF-α is degraded more rapidly than EGF. Other studies have shown that TGF-α, but not EGF, is degraded by the insulin-degrading enzyme (Garcia et al. 1989, Gehm and Rosner 1991). Whether these mechanisms are occurring in the astroglial culture system is unknown.

EGF stimulates the synthesis of IGF-I in astroglia (Han et al. 1992, Chernausek 1993). EGF potentiates IGF induced stimulation of DNA synthesis (Han et al. 1992). The EGF induced increase in IGF-I may also act to

regulate IGFBP levels as was demonstrated previously (Chapter 3). Whether TGF-α has a similar effect on the synthesis of IGF-I in astroglial cells is unknown.

4.5.3 EGF and TGF-α differentially regulate IGFBP-2 and IGFBP-3 synthesis in other cell types

Few studies have examined the regulation of IGFBPs by EGF. Loret et al. (1991) have reported that EGF increased IGFBP-3 and IGFBP-2 levels in the conditioned media of astroglial cells in agreement with our results. However, they used only a single concentration of EGF and analyzed the conditioned media after 5 days in culture. EGF has also been reported to increase the levels of IGFBP-3 in the conditioned media of Swiss 3T3 cells (Corps and Brown 1991). Differential effects of EGF on the regulation of IGFBP-2 and IGFBP-3 have been observed in other cell systems. EGF increases IGFBP-3 levels, but not IGFBP-2, in porcine granulosa cells (Mondschein et al. 1990) and ovine thyroid cells (Eggo et al. 1991). Tissue specific effects may account for the differential regulation of IGFBPs in response to EGF that is observed in other cell systems.

4.5.4 Acidic FGF and basic FGF regulate the biosynthesis of IGFBP-2 and IGFBP-3

The effects of acidic FGF and basic FGF on IGFBP-2 and IGFBP-3 protein levels in the conditioned media were similar. IGFBP-2 was slightly increased by acidic FGF and basic FGF and acidic FGF was more potent than basic FGF. IGFBP-3 protein levels were also increased by both acidic FGF and basic FGF in a dose dependent manner and appeared to have similar potencies.

Both acidic FGF and basic FGF increased stable IGFBP-2 mRNA levels at 24 h. The time course of induction of IGFBP-2 mRNA levels was also similar for both acidic FGF and basic FGF, with a maximum peak occurring at 6 h. Acidic FGF and basic FGF similarly stimulated a dose dependent increase in stable IGFBP-3 mRNA levels with a maximum observed at 25 ng/ml. The time course of IGFBP-3 was slightly different for acidic FGF and basic FGF. Basic FGF stimulated a rapid increase of IGFBP-3 mRNA that peaked at 6 h while acidic FGF also stimulated a rapid increase which peaked at 2 h. These differences in the response of IGFBP stable mRNA to acidic FGF and basic FGF suggest that their effects are specific and not due to a general stimulation of protein synthesis.

These results suggested that the observed increase of IGFBPs in the conditioned media may be due partly to increased levels of stable IGFBP mRNAs. Whether the observed increase in stable mRNA levels was due to increased transcription or increased stability was not examined. The increased levels of IGFBP-2 and IGFBP-3 protein detected in the conditioned media may also be due to mechanisms other than increased stable mRNA levels, such as increased translation, increased rate of secretion of IGFBPs, release of cell surface associated IGFBPs, or a decrease in degradation of IGFBPs.

4.5.5 Possible mechanisms for the more potent effect of basic FGF

The results indicated that acidic FGF and basic FGF have very similar effects on IGFBP-2 and IGFBP-3 biosynthesis both at the protein level and stable mRNA level, however, basic FGF was more potent than acidic FGF in increasing the stable mRNA levels. One possible reason for the greater potency of basic FGF could be due to the presence of structurally variant

isoforms of the FGFR1 that are generated by alternative splicing. A variant of FGFR1 that is generated by alternative exon usage for the third IgG domain encodes a FGFR1 protein that has greater affinity for basic FGF than acidic FGF (Johnson and Williams 1993). The FGFR1 has been detected in astroglial cells, however, the specific isoform(s) have not been characterized (Araujo and Cotman 1992, Logan et al. 1992).

Alternatively, basic FGF could have more potent effects due to its stimulation of IGF-I or IGFR1 mRNA. In a number of cell systems basic FGF increases the level of IGF-I mRNA and peptide (Rosenthal et al. 1991, Drago et al. 1991). An increase in IGF-I or IGF1R could have an additive effect on the biosynthesis of astroglial IGFBPs as was demonstrated previously (Chapter 3). However, the effect of basic FGF on the IGF system in astroglial cells is unclear as two reports have indicated conflicting results. Loret et al. (1991) demonstrated that basic FGF was mitogenic for astroglial cells but did not change IGF-I or IGF1R expression. In contrast, Pons and Torres-Aleman (1992) demonstrated that bFGF increased both IGF-I and IGF1R expression in hypothalamic astroglial cultures. The different effects may be due to region specific differences in the astroglial cultures studied. The effect of basic FGF on IGF expression in our astroglial culture system is unknown.

Differential interaction with low affinity receptors or heparin sulfate proteoglycans may also account for differential effects of basic FGF or acidic FGF. The binding specificity of heparin sulfate proteoglycans has been shown to be modulated during development (Nurcombe et al. 1993). Astroglial cells may express a form of heparin sulfate proteoglycans with preferential affinity for either acidic or basic FGF. The interaction of FGFs with the low affinity receptor is essential for the interaction of the FGFs with the high affinity, signal transducing receptor and therefore may constitute another level of

regulation responsible for the differential effects observed with acidic FGF and basic FGF.

4.5.6 Acidic FGF and basic FGF differentially regulate IGFBP-2 and IGFBP-3 synthesis in other cell types

Few studies have investigated the regulation of IGFBPs by acidic FGF and basic FGF. In newborn olfactory bulb cultures, basic FGF was found to increase IGFBP-2 mRNA and protein levels alone or in combination with IGF-I (Russo et al. 1994). In contrast to our results, Pons and Torres-Aleman (1992) demonstrated that basic FGF decreased both IGFBP-2 and IGFBP-3 protein levels detected in the conditioned media of hypothalamic astroglial cultures. However, in agreement with our results, Loret et al. (1991) demonstrated that basic FGF and acidic FGF increased the level of IGFBP-2 and IGFBP-3 detected in the conditioned media of astroglial cultures.

4.6 CONCLUSIONS

This study has demonstrated that one component of the IGF system, the IGFBPs, are regulated by different growth factors that are expressed within the developing CNS. Moreover, there appears to be differential effects of the various growth factors on the regulation of astroglial IGFBPs even among members of the same growth factor family suggesting that the observed changes in IGFBPs were specific. In order to fully understand the interaction of these growth factors with the IGF system, their regulation on IGF and IGF receptors will need to be determined. In addition, the expression of various components of the EGF family and FGF family of growth factors will also have to be determined. These results do suggest that astroglial cell growth

may be regulated by a complex coordinated interaction between locally produced growth factors in an autocrine or paracrine manner.

CHAPTER FIVE

ALTERATIONS IN THE SYNTHESIS OF INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS AND INSULIN-LIKE GROWTH FACTORS IN C6 GLIOMA CELLS TRANSFECTED WITH A GAP JUNCTION CONNEXIN43 cDNA

5.1 INTRODUCTION

5.1.1 Gap Junctions: Function and Structure

Gap junctions are transmembrane channels that function as a direct means of transfer of small molecules and ions between the cytoplasm of adjacent cells (Lowenstein 1979). Molecules less than 1 kDa are able to pass freely through gap junctions. For example, second messengers of signal transduction, such as cyclic AMP, inositol triphosphate, and calcium, pass freely, while proteins and nucleic acids are excluded. In certain cell types, gap junctions aid in the buffering capacity of these cells by allowing the transport of H^+ or K^+.

Each opposing membrane contributes a hemichannel or connexon, which are composed of six subunits of the gap junction protein connexin. To date, cDNAs from at least 12 different connexin genes have been cloned in the rodent (Saez et al. 1993). A nomenclature has been adopted whereby the connexins are named according to the predicted molecular weight from cDNA sequences. The connexin gene family members possess similar gene structures and have about 50% sequence identity at the amino acid level. The amino- and carboxyl-termini are both cytoplasmic, they are separated by four membrane spanning domains, with a cytoplasmic loop and two extracellular loops (Bennett et al. 1991). The membrane spanning regions and the extracellular loops are highly conserved among the different connexins suggesting that these regions are important for the formation of the channels.

The functional state of a gap junction is determined by the fraction of time it spends in the open versus closed state. Gating of gap junctions is regulated by intracellular free H^+ and/or Ca^{2+} concentration, lipophilic agents, trans-junctional or inside-outside gradient voltage, and

phosphorylation of the connexins (Saez et al. 1993). Functional diversity can be achieved by the formation of heterologous gap junctions where each hemichannel is composed of different connexins. Whether different connexins can associate in one hemichannel is unknown.

5.1.2 Developmental expression of connexin genes with particular reference to the brain

The different connexins have diverse and sometimes overlapping patterns of tissue distribution (Dermietzel and Spray 1993). During development, gap junctional communication is thought to play an important role in organogenesis by allowing the passage of developmental factors or their second messengers, and maintaining developmental compartments by generating boundaries of intercellular communication (Saez et al. 1993).

Connexins26, 32, and 43 have been detected in the developing brain and show specific regional and temporal patterns of expression (Nagy et al. 1992, Yamamoto et al. 1992, Belliveau et al. 1991). Connexin26 is expressed in the rodent brain as early as embryonic day 12, while connexin32 is not detected. This pattern changes postnatally when connexin32 becomes expressed and connexin26 expression is confined to non-neuronal elements (Dermietzel 1989). Connexin43 is the most abundant connexin in the brain and is found predominantly in astroglial cells but also the leptomeninges, endothelial cells and ependyma (Dermietzel and Spray 1993, Naus et al. 1991). Connexin32 is found predominantly on oligodendroglial cells but also in some neuronal subpopulations in the brainstem, cerebral cortex, and basal ganglia (Dermietzel 1989, Dermietzel and Spray 1993).

5.1.3 Relationship between gap junctional intercellular communication and growth

Gap junctional intercellular communication has been proposed to be an important regulator of tissue homeostasis, cellular growth and development. (Lowenstein 1979, Guthrie and Gilula 1989). Aberrant expression of gap junctions has been demonstrated in a diverse array of tumourigenic cells (Klaunig and Ruch 1990). For example, primary rat astroglial cells in culture abundantly express connexin43 in contrast to rat C6 glioma cells which express significantly lower levels (Naus et al. 1991). Dye coupling studies have demonstrated a lower incidence of intercellular communication via gap junctions in C6 cells which suggests a functional correlation to the reduced levels of connexin43.

The proliferative capacity of C6 cells can be reduced by stable transfection with a connexin43 cDNA which results in increased gap junctions on the cell surface and more efficient dye coupling (Zhu et al. 1991). One clone demonstrated a significant reduction in growth, while two other clones, which displayed a lower level of expression of connexin43 and efficient dye coupling, had reduced growth rates that were not significantly different from the wild type cells. Conditioned medium from the connexin43 transfected cells inhibited the growth of the parent C6 cells suggesting that the reduced proliferative capacity of the transfected cells was mediated, at least in part, by a soluble factor released into the conditioned media (Zhu et al. 1992). The nature of this "growth inhibitory factor" is presently unknown. It was hypothesized that the reduced proliferative capacity of C6 cells over-expressing the connexin43 gene may be due partly to alterations in the synthesis of IGFBPs or IGFs.

5.2 OBJECTIVE

The objective of this study was to determine whether the expression of IGFBPs and IGFs in the connexin43 transfected clones was altered and if so, whether these alterations might contribute to the reduced proliferative capacity of the connexin43 transfected C6 cells.

5.3 METHODS

5.3.1 Cell lines and maintenance

Primary astroglia were cultured and maintained as described in section 2.2.1. C6 cells and three clones transfected with a connexin43 cDNA, a high expressing line (Cx43-13) and two intermediate expressing lines (Cx43-12 and Cx43-14), were maintained as described in section 2.2.2.

5.3.2 Northern blot analysis

Total RNA was extracted from primary astroglia, C6 cells, and the three clones transfected with a connexin43 cDNA and subjected to Northern blotting as described in section 2.5. The Northern blot was probed with a [^{32}P]labeled cDNAs for connexin43, IGFBP-2, IGFBP-3, IGFBP-4, IGF-I, IGF-II and 18 S ribosomal RNA.

5.3.3 Immunocytochemistry

C6 glioma and Cx43-13 cells were cultured on glass coverslips coated with poly-L-lysine, fixed in 95% ethanol/5% glacial acetic acid (v/v) for 20 min at -20°C, rinsed with PBS, and incubated for 2 h in a 1:500 dilution of rabbit polyclonal connexin43 antiserum raised against a synthetic peptide

corresponding to amino acids 302-319 of the predicted amino acid sequence (a gift from Dr. B. Nicholson, SUNY, Buffalo, NY). Control slides were incubated in a pre-immune serum (1:500) from the same rabbit. This was followed by incubation in fluorescein-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) diluted 1:50. Coverslips were then rinsed in PBS and mounted on slides in PBS containing 50% (v/v) glycerol and 0.1% *p*-phenylenediamine (Zhu et al. 1991).

5.3.4 Ligand blot analysis

Conditioned media from wild type C6 glioma cells and the three clones transfected with a connexin43 cDNA, Cx43-12, Cx43-13, and Cx43-14, were collected and analyzed by ligand blotting as described in section 2.3.

To detect the presence of IGFBP protease activity, increasing amounts of CM from Cx43-13 cells (10 µl to 50 µl) were co-incubated with a constant amount of CM (50 µl) from primary astroglial cells and wild type C6 glioma cells (20% to 50%, v/v) at 37°C for 1 h prior to analysis by ligand blotting.

5.3.5 [^{3}H]thymidine incorporation

The wild type C6 cells and the clone Cx43-13 were treated with IGFs or conditioned media from the clone Cx43-13 and the incorporation of [^{3}H]thymidine was assayed as described in section 2.12.

5.4 RESULTS

5.4.1 Detection of connexin43 mRNA and protein

Figure 5.1 A shows the Northern blot analysis of total RNAs from primary astroglia, C6 glioma cells, and the transfected cell lines Cx43-13, Cx43-12 and Cx43-14, probed with a radiolabeled connexin43 cDNA. A 3.0 kb endogenous connexin43 mRNA was readily detectable in all of the glial cell types except the wild type C6 glioma cells, in which it was present in low abundance. An encoded 2.25 kb mRNA from the transfected connexin43 cDNA was also present in all of the transfected cell lines, Cx43-13, Cx43-12 and Cx43-14. Densitometric quantification revealed that the high expressing clone Cx43-13 contained a 50 fold greater abundance of total connexin43 mRNA than the parent C6 cell line. Clone Cx43-13 had a 5 fold greater level of the 2.25 kb connexin43 mRNA than intermediate expressing clones Cx43-12 and Cx43-14 (Figure 5.1 A).

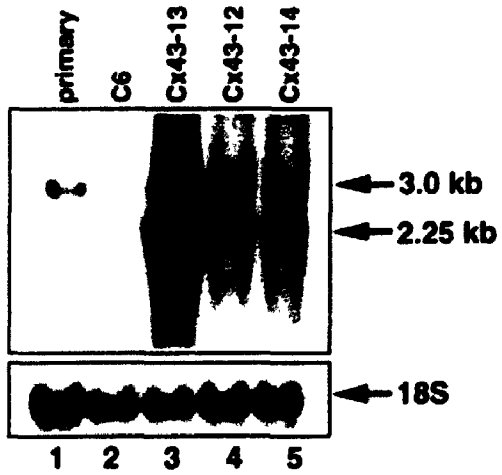
Immunocytochemistry was used to demonstrate that the connexin mRNA was translated and that the protein was localized to areas of cell-cell contact. Immunocytochemically identifiable connexin43 protein was demonstrated on the cell surfaces and intracellularly of the connexin43 transfected clones (Figure 5.1 B). The greater abundance of the connexin43 mRNA in the transfected cells was associated with a greater amount of immunoreactive connexin43 protein. In addition, these gap junctions were proven to be functional by dye transfer studies (Zhu et al. 1992).

5.4.2 Detection of IGFBPs

Ligand blot analysis of conditioned media from primary astroglia, C6 cells and the three clones transfected with a connexin43 cDNA is shown in

Figure 5.1 (**A**) Northern blot analysis of total RNAs (20 μg/lane) from primary astroglia (lane 1) C6 glioma (lane 2) Cx43-13 (lane 3), Cx43-12 (lane 4) and Cx43-14 (lane 5) hybridized with a [^{32}P]connexin43 cDNA. A 3.0 kb endogenous connexin43 mRNA was readily detectable in primary astroglia (lane 1) and the connexin43 transfected clones Cx43-13, Cx43-12 and Cx43-14 (lanes 3-5), but almost undetectable in the C6 glioma (lane 2). The 2.25 kb mRNA encoded from transfected connexin43 cDNA was detected in great abundance in Cx43-13 (lane 3), but in lower abundance in Cx43-12 (lane 4) and Cx43-14 (lane 5). (**B**) Immunocytochemical analysis of the presence of connexin43 gap junction protein in primary astroglia (left panel), wild type C6 glioma cells (middle panel) and Cx43-13 cells (right panel). Very little immunofluorescence was observed in C6 cells, whereas connexin43 immunoreactivity was evident throughout the Cx43-13 cells (both cell membrane and cytosolic).

A



B

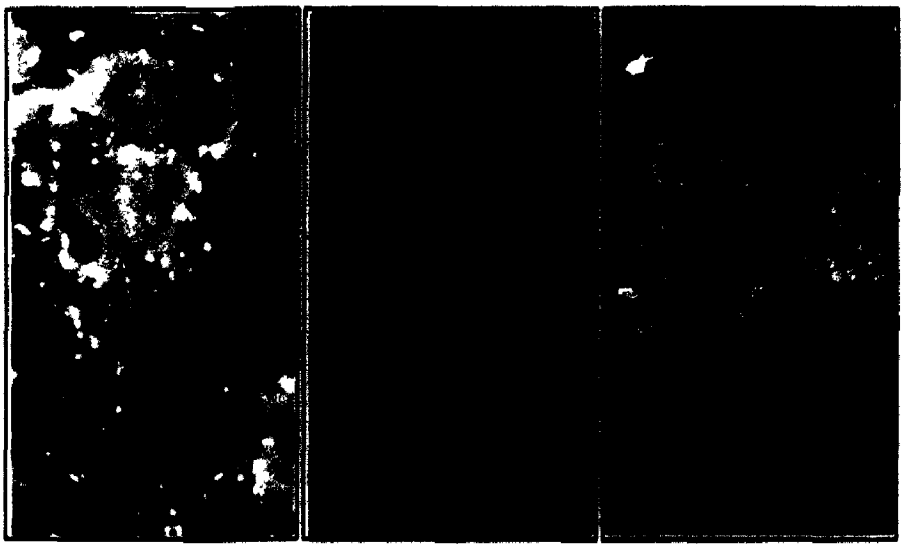


Figure 5.2. Primary astroglia secreted two IGFBPs of M_r 34 kDa and 40-45 kDa, previously identified as IGFBP-2 and IGFBP-3 respectively (Chapter 3), and wild type C6 glioma cells secreted three IGFBPs of M_r 40-45 kDa (IGFBP-3), 34 kDa (IGFBP-2) and 24 kDa. The 24 kDa protein, although not identified immunologically because of the lack of a specific antiserum, was deduced to be IGFBP-4 based on its molecular size and the detection of IGFBP-4 mRNA transcripts by Northern blot analysis (see below). C6 cells secreted significantly greater amounts of IGFBP-3 and lesser amounts of IGFBP-2 compared to primary astroglia.

Analysis of CM from the three clones transfected with a connexin43 cDNA, revealed significant differences in the profiles of IGFBPs secreted between the high expressing Cx43-13 line and the intermediate expressing lines (Cx43-12 and Cx43-14) (Figure 5.2). Cx43-13 cells secreted little or no IGFBP-3, whereas the Cx43-12 and Cx43-14 cells secreted IGFBP-3 in quantities relatively similar to the wild type C6 cells. The three clones transfected with a connexin43 cDNA secreted IGFBP-2 in quantities similar to C6 cells but much lower than primary astroglia. In contrast, the Cx43-13 cells produced a large amount of the 24 kDa IGFBP whereas the C6, Cx43-12 and Cx43-14 cells produced little or none.

5.4.3 Analysis of Cx43-13 conditioned medium for proteolytic degradation fragments of IGFBP-3

To determine if the lack of IGFBP-3 and the presence of the 24 kDa IGFBP in the CM of Cx43-13 cells was due to proteolytic degradation of IGFBP-3, increasing concentrations of Cx43-13 conditioned medium were incubated at 37°C with CM from C6 cells or primary astroglia (Figure 5.3.). This procedure did not reduce the amount of IGFBP-3 in the CM of both C6 and

Figure 5.2 Ligand blot analysis of CM from primary astroglia, C6 glioma cells, and the C6 clones transfected with the connexin43 cDNA (Cx43-13, Cx43-12 and Cx43-14) using [^{125}I]IGF-II as the radioligand. Lane 1: primary astroglia, lane 2: C6 glioma cells, lane 3: clone Cx43-13, lane 4: clone Cx43-12, lane 5: clone Cx43-14.

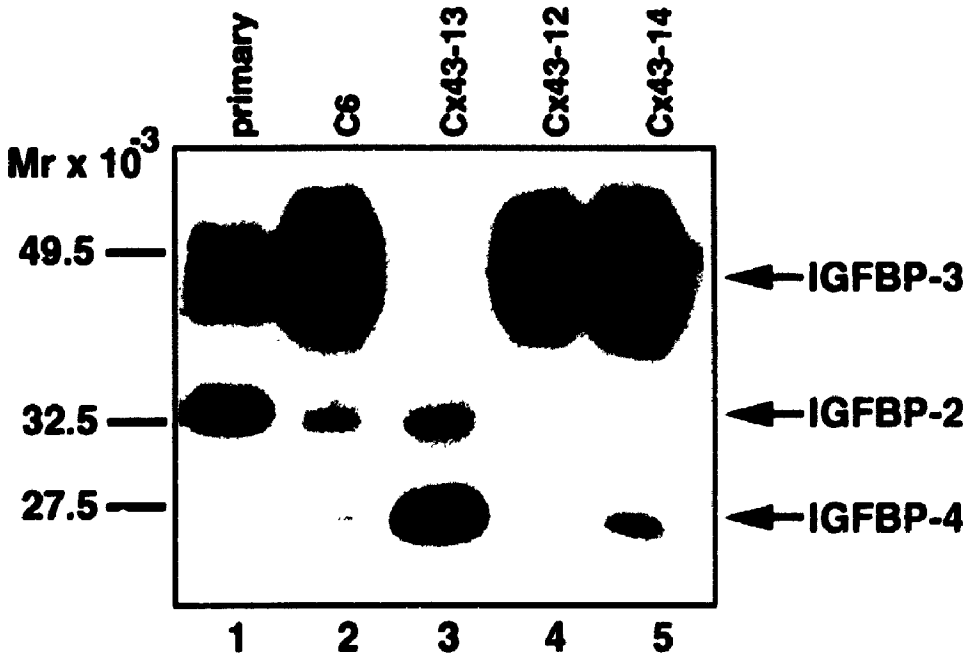
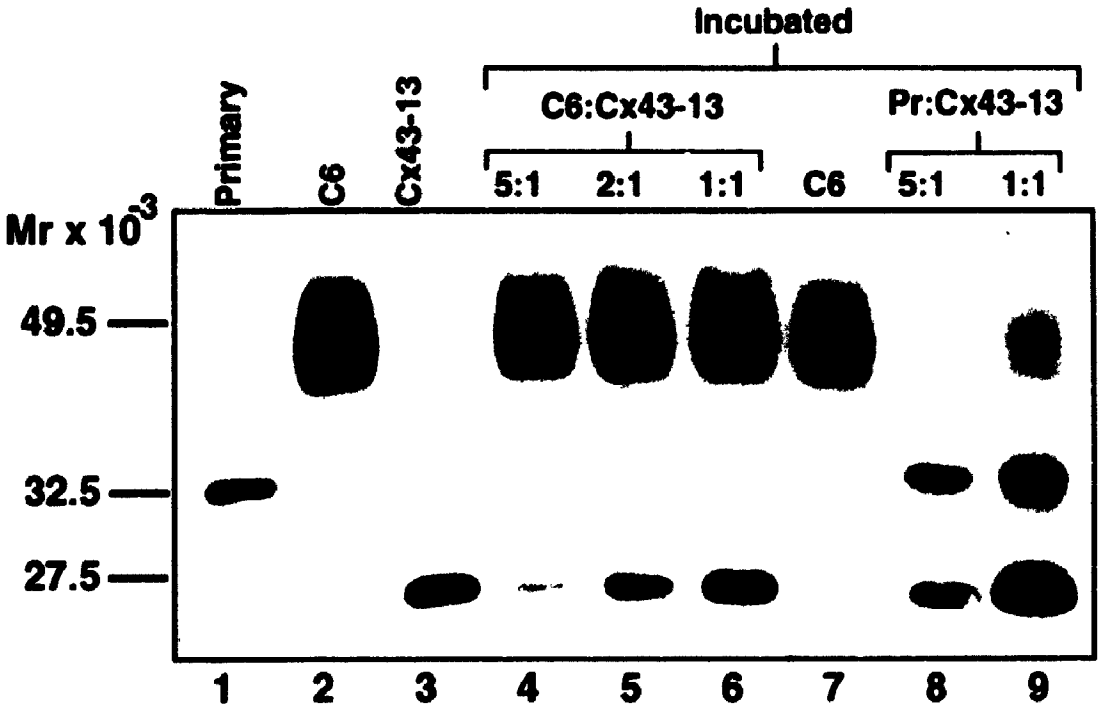

primary
C6
Cx43-13
Cx43-12
Cx43-14
Mr x 10^3
49.5
32.5
27.5
IGFBP-3
IGFBP-2
IGFBP-4
1
2
3
4
5

Figure 5.3 Ligand blot analysis of CM from different glial cells co-incubated at 37°C for 1 h. [^{125}I]IGF-II was used as the radioligand. Unincubated CM from C6 glioma cells 50 µl (lanes 2 and 7) and primary astroglia 50 µl (lane 1) are shown for comparison. Incubation of CM from C6 cells (50 µl) with increasing volumes of Cx43-13 CM 10 to 50 µl (lanes 4-6) or CM from primary astroglial cells (50 µl each lane) with increasing volumes of Cx43-13 CM 10 and 50 µl (lanes 8-9) did not reveal a reduction in the amount of IGFBP-3. The gradual increases in IGFBP-4 (lanes 4-6 and 8-9) were most likely due to the increasing volumes of Cx43-13 CM added.


Incubated
C6:Cx43-13
Pr:Cx43-13
Primary
C6
Cx43-13
5:1
2:1
1:1
C6
5:1
1:1
Mr x 10^3
49.5
32.5
27.5
1
2
3
4
5
6
7
8
9

primary astroglia, indicating that there was no significant IGFBP-3 specific protease activity in Cx43-13 CM. The increasing amounts of the 24 kDa IGFBP observed in the lanes co-incubated with Cx43-13 CM were likely due to the increasing volumes of Cx43-13 CM added in the incubation, and not due to degradation products of IGFBP-3.

5.4.4 Detection of IGFBP mRNAs

Northern analysis (Figure 5.4) revealed abundant IGFBP-2 stable mRNA in the primary astroglia, with little detected in the C6 and the three clones transfected with a connexin43 cDNA. IGFBP-3 mRNA was detected in primary astroglia, C6 and intermediate expressing clones Cx43-12 and Cx43-14, but not in the high expressing clone Cx43-13. IGFBP-4 mRNA was detected in significant levels only in clone Cx43-13. IGFBP-5 or -6 mRNAs were not detected suggesting that the 24 kDa IGFBP was most likely IGFBP-4. The identity and amount of IGFBP mRNA transcripts detected by Northern blotting corresponded to the amount of IGFBP protein detected by ligand blotting.

5.4.5 The concentration of IGFs in the conditioned media

The radioimmunoassayable concentration of IGF-I and IGF-II in the 24 h CM of C6 and the three clones transfected with a connexin43 cDNA are shown in Table 5.1. IGF-II levels in the CM from both the C6 and transfected cells were below the detection limits of the radioimmunoassay. In contrast, the IGF-I concentration measured in the CM of C6 cells was 1.36 $\pm$ 0.41 ng/ml (range 0.4-2.1 ng/ml). The transfected cells had significantly lower amounts of IGF-I in the CM compared to the wild type C6 cells. Cx43-13 cells, which have the slowest growth rate, secreted the least amount of IGF-I into the

Figure 5.4 Northern blot analysis of total RNA (20 μg/lane) from primary astroglia (lane 1), C6 glioma (lane 2), Cx43-13 (lane 3), Cx43-12 (lane 4) and Cx43-14 (lane 5) probed sequentially with [^{32}P]labeled cDNAs for IGFBP-2 (first panel), IGFBP-3 (second panel), IGFBP-4 (third panel) and 18S ribosomal RNA (fourth panel). The presence and the relative abundance of the IGFBP mRNAs corresponds to the relative amounts of IGFBP proteins detected in the CM.

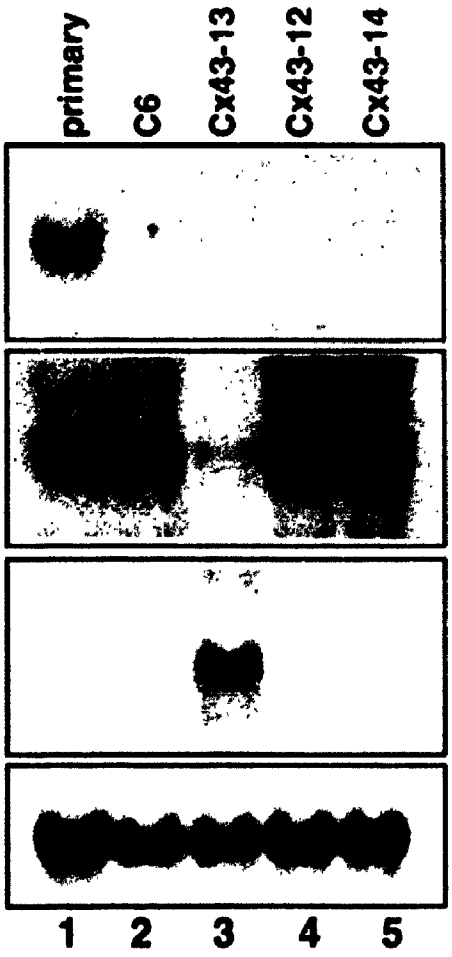

primary
C6
Cx43-13
Cx43-12
Cx43-14
1
2
3
4
5

Table 5.1 The concentration of IGF-I and IGF-II in the 24 h conditioned media of C6 (wild type) and C6 cells transfected with connexin43 cDNA determined by radioimmunassay. Values show mean ± SEM (n=3). ND = Not detectable; below the detection limit of the radioimmunoassay of 1.3 ng/ml, * indicates a statistically significant change ($p < 0.05$).

Cell Type	IGF-I ng/ml (Range)	IGF-II ng/ml
C6	1.36 ± 0.41 (0.4 - 2.1)	ND
Cx43-13	0.11 ± 0.06* (0.06 - 0.2)	ND
Cx43-12 and Cx43-14	0.26 ± 0.1* (0.1 - 0.5)	ND

medium, but concentrations in the CM were not different statistically from those in the CM of Cx43-12 and -14 cells.

5.4.6 Detection of stable IGF-I and IGF-II mRNA levels

A relatively high level of IGF-I stable mRNA was detected in the wild type C6 glioma cells (Figure 5.5). A major 7.4 kb transcript was detected in addition to minor transcripts of 4.0, 2.0 and 1.2 kb. IGF-I mRNA was not detected in the primary astroglia on this autoradiograph, however with longer exposure times, IGF-I mRNA transcripts were detectable (not shown). The IGF-I stable mRNA levels were reduced in all of the three clones transfected with a connexin43 cDNA compared to the wild type C6 glioma cells. IGF-I mRNA was very low in clones Cx43-12 and Cx43-14 and was barely detectable in clone Cx43-13. IGF-II stable mRNA was not detected (data not shown).

5.4.7 Alterations in the incorporation of [^{3}H]thymidine in C6 glioma cells and clone Cx43-13

To determine whether the increase in gap junctional communication by increased expression of connexin43 would result in increased responsiveness to the mitogenic stimulation of IGFs, C6 and clone Cx43-13 were treated with varying concentrations of IGFs and [^{3}H]thymidine incorporation was assayed (Figure 5.6). C6 glioma cells did not respond to the addition of varying doses of IGF-I or IGF-II, as the incorporation of [^{3}H]thymidine was unchanged from control conditions in SFM. In contrast, clone Cx43-13 responded to IGFs in a dose dependent manner. The highest stimulation of incorporation of [^{3}H]thymidine was observed at an ICF-II

Figure 5.5 Northern blot analysis of total RNA (20 μg/lane) from primary astroglia (lane 1), C6 glioma (lane 2), Cx43-13 (lane 3), Cx43-12 (lane 4) and Cx43-14 (lane 5) probed sequentially with [^{32}P]labeled cDNAs for IGF-I (upper panel) and 18S ribosomal RNA (lower panel). The presence and the relative abundance of the IGF-I mRNAs corresponds to the relative amounts of IGF-I proteins detected in the CM by RIA.

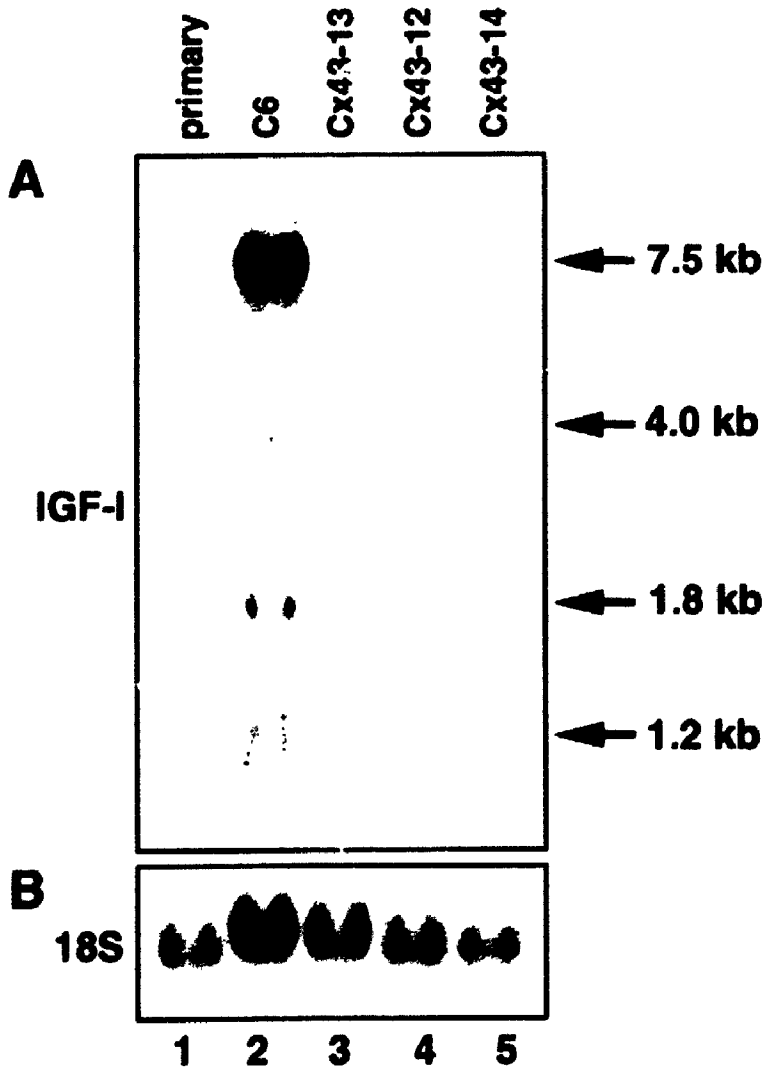
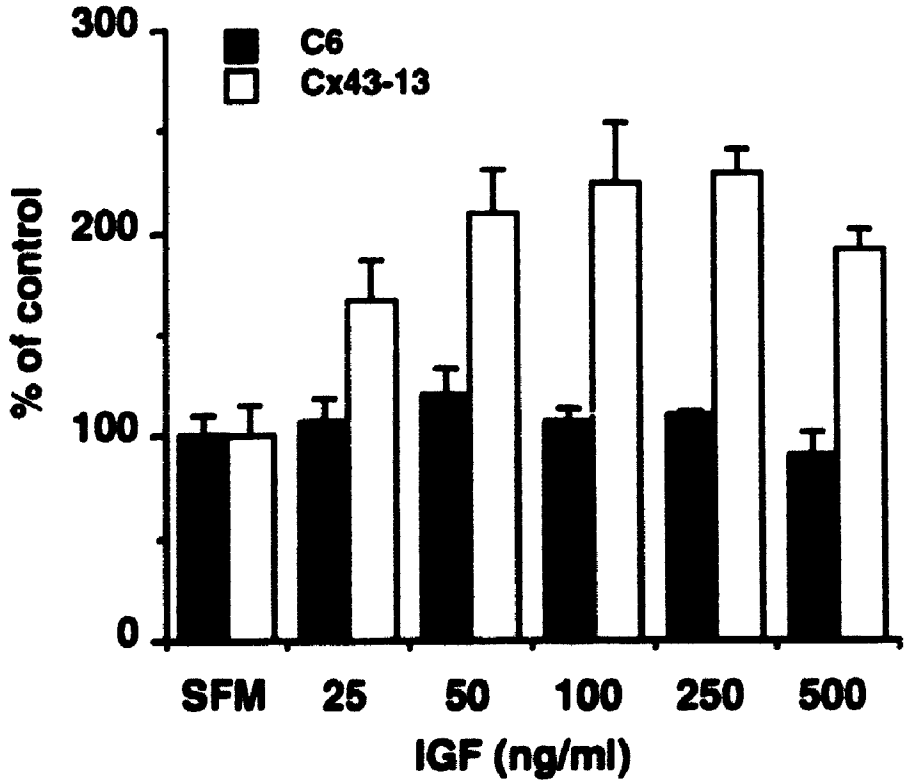

primary
C6
Cx43-13
Cx43-12
Cx43-14
A
IGF-I
7.5 kb
4.0 kb
1.8 kb
1.2 kb
B
18S
1
2
3
4
5

Figure 5.6 [^{3}H]thymidine incorporation of C6 glioma cells in response to increasing concentrations of either IGF-I or IGF-II (■) and Cx43-13 cells to IGF-II (❑). Values are expressed as percent of control (SFM condition) [mean ± SEM] (n=4). Cx43-13 cells responded to IGF-II in a dose dependent manner, with maximal stimulation occurring at 250 ng/ml. A similar dose reponse was achieved with IGF-I (data not shown).


300
200
100
0
% of control
C6
Cx43-13
SFM
25
50
100
250
500
IGF (ng/ml)

concentration of 250 ng/ml resulting in a greater than 2-fold stimulation above control.

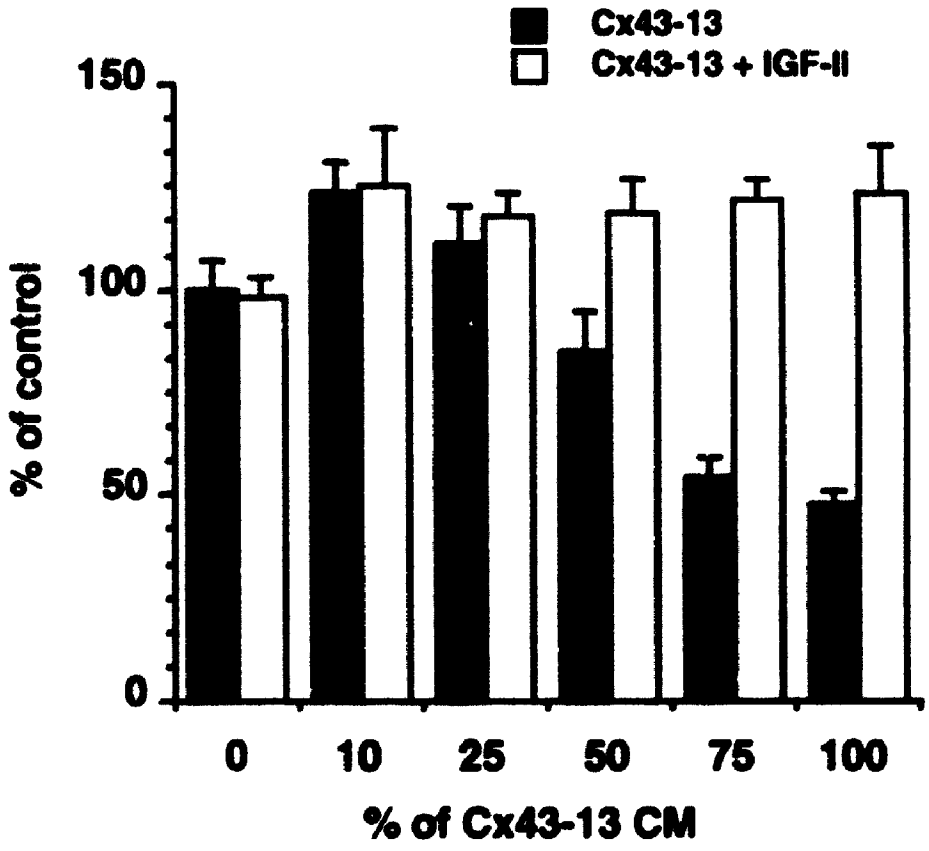
When C6 glioma cells were incubated with CM from Cx43-13 cells, incorporation of [^{3}H]thymidine was reduced (Figure 5.7). A 50% decrease from control was observed in conditions where C6 cells were treated with 75% to 100% of clone Cx43-13 CM. A slightly stimulatory response was observed at 10% clone Cx43-13 CM. Addition of the same concentrations of CM from C6 cells did not alter [^{3}H]thymidine incorporation of C6 cells (data not shown), indicating that the reduction in DNA synthesis observed with Cx43-13 CM was not due to the alteration of nutrients in the incubating media. The addition of IGF-II (100 ng/ml) alone did not stimulate the wild type C6 cells however, IGF-II effectively reversed the decrease in [^{3}H]thymidine incorporation observed upon the addition of Cx43-13 CM.

5.5 DISCUSSION

5.5.1 The IGFBP profile changes in response to different levels of expression of the connexin43 cDNA

This study demonstrates that different types of glial cells with variable expression of the connexin43 cDNA, and differing growth patterns, secrete different profiles of IGFBPs. Primary astroglia secrete IGFBP-2 and IGFBP-3, a finding in agreement with previous reports from our laboratory and others (Han et al. 1988b, Ocrant et al. 1990, Ocrant et al. 1989, Olson et al. 1991). C6 glioma cells synthesize greater amounts of IGFBP-3 and lesser amounts of IGFBP-2 compared to the primary astroglial cells. C6 glioma cells exhibit a

Figure 5.7 [^{3}H]thymidine incorporation of C6 glioma cells in response to an increasing percentage of Cx43-13 CM without (■) and with IGF-II (100 ng/ml) (❑). Values are expressed as percent of control (SFM condition) [mean ± SEM] (n=4). Cx43-13 CM decreased [^{3}H]thymidine incorporation to 50% of control at 75% Cx43-13 CM. IGF-II reversed the decrease in [^{3}H]thymidine incorporation. Incubation with similar concentrations of C6 cell CM did not alter the [^{3}H]thymidine incorporation of C6 cells, indicating that the Cx43-13 CM effect was not due to the alterations in nutrients in the incubating medium.


Cx43-13
Cx43-13 + IGF-II
150
100
50
0
% of control
0
10
25
50
75
100
% of Cx43-13 CM

decrease in intercellular communication via gap junctions as compared to primary astroglia, as demonstrated by dye coupling studies (Naus et al. 1991).

Intercellular communication was increased in C6 cells by stable transfection of the connexin43 cDNA, and was accompanied by a decrease in the growth rate (Zhu et al. 1991). Ligand blot analysis of the conditioned media of the three clones transfected with a connexin43 cDNA demonstrated that the IGFBP profiles were altered. The clone Cx43-13, which expressed the highest levels of connexin43 mRNA, showed the greatest level of dye coupling and the slowest growth rate (Zhu et al. 1991), exhibited the most dramatic change in IGFBP synthesis. In these cells, the synthesis of IGFBP-3 was completely abolished and the synthesis of IGFBP-4 was significantly increased. These changes were accompanied by parallel alterations in the abundance of the IGFBP-3 and -4 mRNAs. The IGFBP profiles in the intermediate expressors Cx43-12 and Cx43-14 were not dramatically different from those of the parent C6 cells.

The co-incubation studies demonstrated that the lack of IGFBP-3 in CM from Cx43-13 cells was not due to the presence of IGFBP-3 specific proteases which have been shown to be present in the sera of pregnant mothers (Hossenlopp et al. 1990, Giudice et al. 1990, Davenport et al. 1992a) and in patients with severe illness (Davies et al. 1991). A specific protease for IGFBP-4 has also been described which can be induced by IGF-I (Fowlkes and Freemark 1992, Myers et al. 1993b, Cheung et al. 1994). Failure to decrease the levels of IGFBP-4 in the CM of Cx43-13 cells co-incubated with CM from primary astroglia and wild type C6 glioma cells indicates that the latter cells do not produce this protease, and therefore it is unlikely that this protease plays any role in altering IGFBP-4 levels.

5.5.2 The synthesis of IGF-I is altered in response to expression of the connexin43 cDNA

The levels of radioimmunoassayable IGF-I determined in the CM of C6 glioma cells were consistent with previous reports (Kiess et al. 1989, Chernausek 1993). IGF-I levels in the CM from the three clones transfected with a connexin43 cDNA were decreased as were the levels of IGF-I stable mRNA, indicating that overexpression of the connexin43 cDNA is associated with a reduction in expression of IGF-I. Trojan et al. (1992) have demonstrated that the reduction of endogenous IGF-I production in C6 cells by transfection of an antisense cDNA, result in the loss of tumourigenicity of C6 cells *in vivo*, suggesting an association between low levels of IGF-I expression and a reduction in growth rate. IGF-II concentrations in the CM from C6 cells was undetectable as reported previously (Kiess et al. 1989). The transfected cells also synthesized similarly low levels of IGF-II into the CM.

5.5.3 Changes in IGF responsiveness in connexin43 transfected clones

In this study, C6 glioma cells did not respond mitogenically to either exogenous IGF-I or IGF-II, as previously demonstrated by Kiess et al. (1989). This lack of effect was not due to the absence of either IGF-I or IGF-II/mannose-6 phosphate receptors, but most likely due to the synthesis of IGF-I to which the C6 cells are responding in an autocrine manner (Kiess et al. 1989). Transfection with the connexin43 cDNA in C6 cells, decreased IGF-I mRNA levels and IGF-I secretion, and may have restored the ability of the cells to respond mitogenically to IGF-I and IGF-II. These findings support the hypothesis of Keiss et al. (1989), that C6 cells respond to endogenous IGF-I in an autocrine manner, and cannot respond to further additions of exogenous IGFs.

Incubation of CM from Cx43-13 with C6 cells resulted in a dose dependent decrease in their DNA synthesis. Zhu et al. (1992) have demonstrated that CM from Cx43-13 resulted in a dose dependent decrease in the cell number of C6 cells over 4 d in culture, suggesting that the inhibitory activity of the Cx43-13 cells was due to a secreted "transferable factor". Cx43-14 CM had an intermediate effect on decreasing the growth of the C6 cells, suggesting that the inhibitory activity was associated with the level of expression of connexin43. IGF-II was able to reverse the decrease in DNA synthesis by Cx43-13 CM on C6 cells, suggesting that the "transferable growth inhibitory factor" could be related to the IGF system.

5.5.4 Possible mechanisms for the alterations of IGF-I and IGFBP expression

Although random insertion could have led to alterations in the expression of IGFBP genes, the fact that various transfected lines exhibited differing IGFBP profiles and all of them synthesize lower levels of IGF-I suggest that the Cx43-13 synthetic characteristics are not artifactual. It is also unlikely that a single random integration of the expression vector pLTRCx43 into the genome of the parent C6 cells could have disrupted IGFBP-3, IGFBP-4 and IGF-I genes since they are all located in different chromosomes (Shimasaki et al. 1990, 1991a, Shimatsu et al. 1987). However, it is possible that the integration has generated certain *trans*-acting element(s) that may influence the expression of these genes. The difference in IGFBP profiles between different stable transfectants may also be related to variations in the site of chromosomal insertion or the number of copies of the expression vector pLTRCx43 integrated into the genome of these various clones (Zhu et al. 1991). Alternatively, an increase in other growth inhibitory factors (e.g.

transforming growth factor-β) or a decrease in other growth promoting factors (e.g. transforming growth factor-α, fibroblast growth factor) (Seroogyi et al. 1991, Pruss et al. 1982, Yamamoto et al. 1991), could be responsible for the changes observed.

The mechanism by which the increase in intercellular communication via gap junctions leads to changes in the synthesis of IGFBPs and IGF-I is unknown. It may result from the increased transfer of micromolecules between cells, that in turn may have direct or indirect effects on the expression of genes encoding growth factors or growth factor binding proteins. Such mechanisms may be operative in regulation of growth of normal cells and may be lost in tumourigenic cells (Lowenstein 1979, Klaunig and Ruch 1990). In a previous study, it was demonstrated that the growth inhibitory property of the Cx43-13 cells is transferable, suggesting that the slow growth rate observed in these cells may be mediated via either an increased production of soluble growth inhibitory factor(s) or a decreased production of growth stimulators (Zhu et al. 1992).

Changes in growth rates have also been observed in other cell types (communication-incompetent SkHep1 hepatoma cell line and 10 T1/2 cells) in which connexin32 and/or connexin43 genes were overexpressed using similar techniques (Eghbali et al. 1990, Fishman et al. 1990, Metha et al. 1991). Whether the IGF or IGFBP profiles were altered similarly in these cells has not been reported.

5.6 CONCLUSION

This study demonstrated that the growth promoting factor, IGF-I, is decreased, and the modulatory IGFBPs are altered in the connexin43 transfected cell lines. IGFBP-4 has been shown to be a potent inhibitor (Mohan et al. 1989, Shimonaka et al. 1989) and IGFBP-3 a potentiator (Blum et al. 1989, Conover et al. 1990) of the mitogenic and metabolic actions of IGFs in other cell lines. It is therefore reasonable to speculate that a decrease in IGF-I, IGFBP-3, or an increase in IGFBP-4 synthesis may at least in part contribute to the change in the growth pattern of C6 cells transfected with a connexin43 cDNA. However, the causal relationship between IGFBPs and IGFs, and the growth pattern of C6 glioma cells awaits further studies. This association between gap junctions, and growth factors and their binding proteins in relation to the proliferative behavior of normal and tumor cells indicates the important roles of cell to cell contact and paracrine factors in the regulation of cellular growth.

CHAPTER SIX

C6 GLIOMA CELLS TRANSFECTED WITH AN IGFBP-2 cDNA: CHARACTERIZATION OF CLONES AND COMPARISON OF GROWTH

6.1 INTRODUCTION

IGFBPs have a wide variety of functions including the transport of IGFs in the circulation and extracellular spaces, prolonging the half-life of IGFs, regulating their clearance, and modulating the actions of IGFs on target tissues. *In vitro* studies have demonstrated that IGFBPs may either inhibit or potentiate the biologic actions of IGFs (DeMellow and Baxter 1988, Elgin et al. 1987, Blum et al. 1989, Conover et al. 1990, Clemmons et al. 1990, Jones et al. 1993a). In addition to their modulation of IGF action, direct functions for IGFBPs independent of IGFs, have been proposed. IGFBP-1 was found to bind specifically to the $\alpha_5\beta_1$ integrin receptor through its Arg-Gly-Asp integrin recognition motif and this binding was correlated with the stimulation of cell migration (Jones et al. 1993b). Overexpression of IGFBP-3 in fibroblast cells derived from mice with a null mutation in the type 1 receptor, resulted in decreased cellular growth, which suggested that overexpression of IGFBP-3 reduced cellular growth independent of IGF action (Valentinis et al. 1995). The addition of exogenous IGFBP-3 inhibited the growth of a human breast cancer cell line independent of IGF stimulation, by interaction with a specific cell surface protein (Oh et al. 1993a, 1993b). However, the mechanism of action of IGFBPs at the cellular level, both in modulating IGF actions and possible direct functions, remains unclear.

We have shown previously that the exogenous addition of IGFBP-2 purified from BRL-? ₁ cells can inhibit the mitogenic action of IGFs on primary astroglial cultures, and that this inhibition is a result of competition for IGF binding to the receptor (Han et al. 1988b). However, exogenous addition of IGFBPs may not accurately reflect the true autocrine/paracrine nature of the IGF system as there is increasing evidence demonstrating the

interaction of IGFBPs with either extracellular matrix (Jones et al. 1993a) or cellular components at the membrane level (Delhanty and Han 1993, Conover et al. 1990, Jones et al. 1993b). The rat C6 glioma cell line was chosen as a model system as it synthesizes IGF-I, expresses IGF receptors (Kiess et al. 1989) and its growth is regulated by IGF-I (Trojan et al. 1992). Primary astroglial cells, which demonstrate regulated growth, synthesize high levels of IGFBP-2, while C6 glioma cells, with tumourigenic growth, express very low levels of IGFBP-2 (Chapter 5).

6.2 OBJECTIVE

The objective of this study was to create a system whereby the endogenous expression of IGFBP-2 in glial cells is altered, and to address the question of whether the overexpression of IGFBP-2 leads to an altered growth phenotype.

6.3 METHODS

6.3.1 Transfection of C6 glioma cells

An ovine IGFBP-2 cDNA, under the control of the constitutive cytomegalovirus promoter was transfected into C6 glioma cells using the polybrene/DMSO technique as described in Section 2.2.3. Nine clones stably expressing the IGFBP-2 cDNA were selected for analysis.

6.3.2 Characterization of the clones of C6 cells transfected with an IGFBP-2 cDNA

Clones were grown until confluent, incubated for 24 h in SFM, and the conditioned media were collected and analyzed by ligand blotting as described in Section 2.3. Total RNA was isolated from the cells and subjected to Northern analysis as described in Section 2.5.

6.3.3 Determination of growth rates

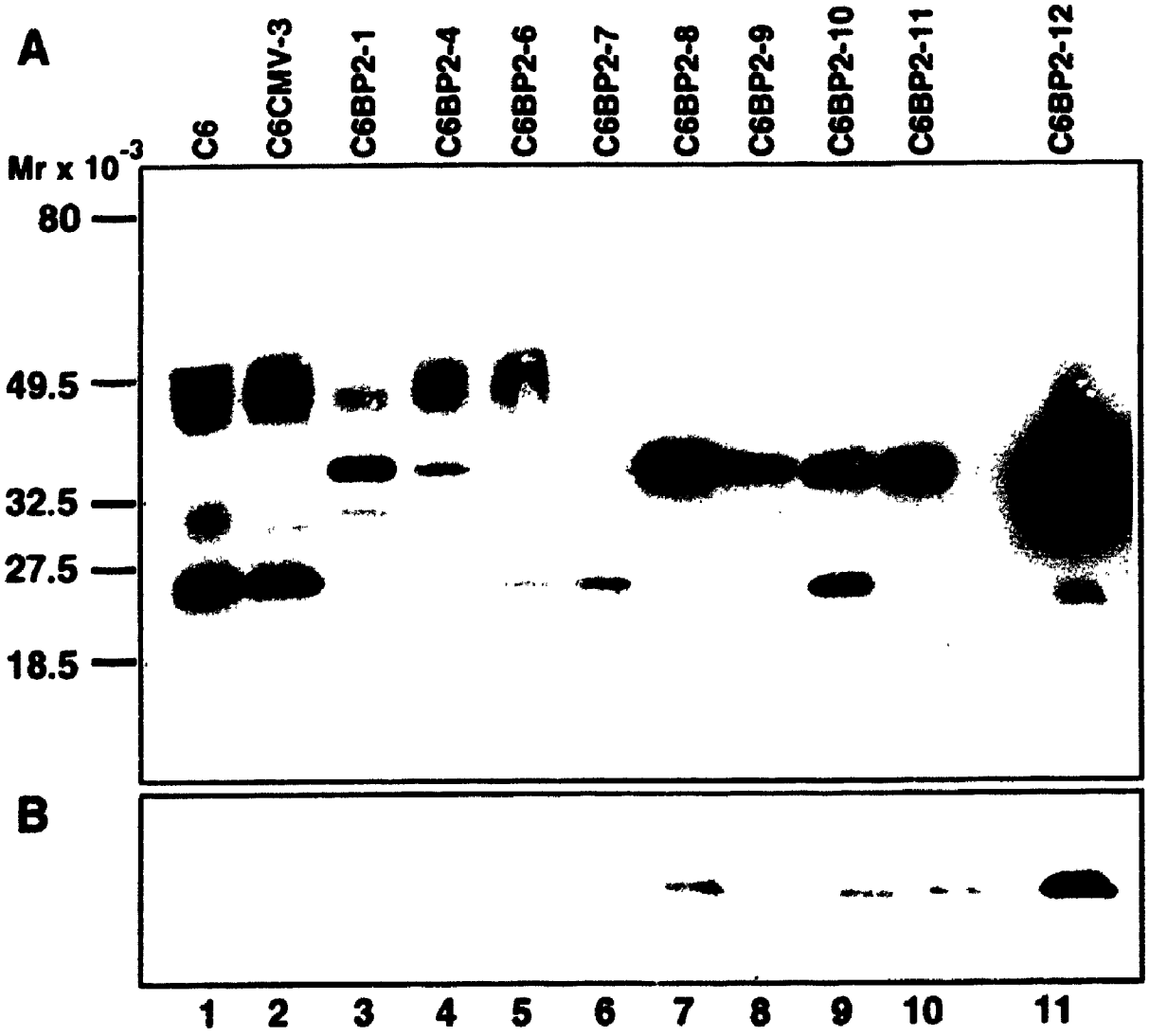
Changes in cell number over 72 h were determined for selected clones as described in Section 2.10. Doubling time for each clone was calculated as the change in time divided by the log (change in cell number). Mean doubling time and SEM were determined from three separate experiments for each clone. Analysis of variance was used to determine statistically significant changes in mean doubling times between clones.

6.4 RESULTS

6.4.1 Detection of IGFBPs in the C6 cells transfected with an IGFBP-2 cDNA

Conditioned media was analyzed by ligand blotting using both radiolabeled IGF-I (Figure 6.1 A) and IGF-II (data not shown). Wild type C6 glioma cells secreted IGFBPs of M_r 40-45, 28 and 22. The 40-45 kDa IGFBP has been identified immunologically as IGFBP-3 (Ocrant et al. 1990, 1991). The 22 kDa IGFBP, although not identified immunologically because of the lack of a specific antiserum, was deduced to be IGFBP-4 based on its molecular size and the detection of IGFBP-4 mRNA in Northern blots. The identity of the 28 kDa IGFBP remains unknown, but may be glycosylated IGFBP-4 based on its

Figure 6.1 (**A**) Ligand blot analysis of CM from wild type C6 glioma cells (lane 1), the pRc/CMV vector transfected clone (lane 2) and the pRc/CMV-oIGFBP-2 transfected clones (lanes 3-11). CM were collected from cells after incubation for 24 h in SFM, subjected to SDS-PAGE, transferred to nitrocellulose, incubated with [^{125}I]IGF-I, and visualized by autoradiography. (**B**) Western blot analysis with an antiserum against bovine IGFBP-2 showing an immunoreactive band of 34 kDa in the pRc/CMV-oIGFBP-2 transfected clones.


A
C6
C6CMV-3
C6BP2-1
C6BP2-4
C6BP2-6
C6BP2-7
C6BP2-8
C6BP2-9
C6BP2-10
C6BP2-11
C6BP2-12
Mr x 10-3
80
49.5
32.5
27.5
18.5
B
1 2 3 4 5 6 7 8 9 10 11

molecular size and the lack of detectable mRNAs for IGFBP-1, -5, and-6. Comparison of IGF-I and IGF-II as radioligands revealed some differences in the ligand binding of different IGFBPs. IGFBP-4 was most readily detectable with IGF-I, while IGFBP-2 and IGFBP-3 were equally detectable using both IGF-I and IGF-II.

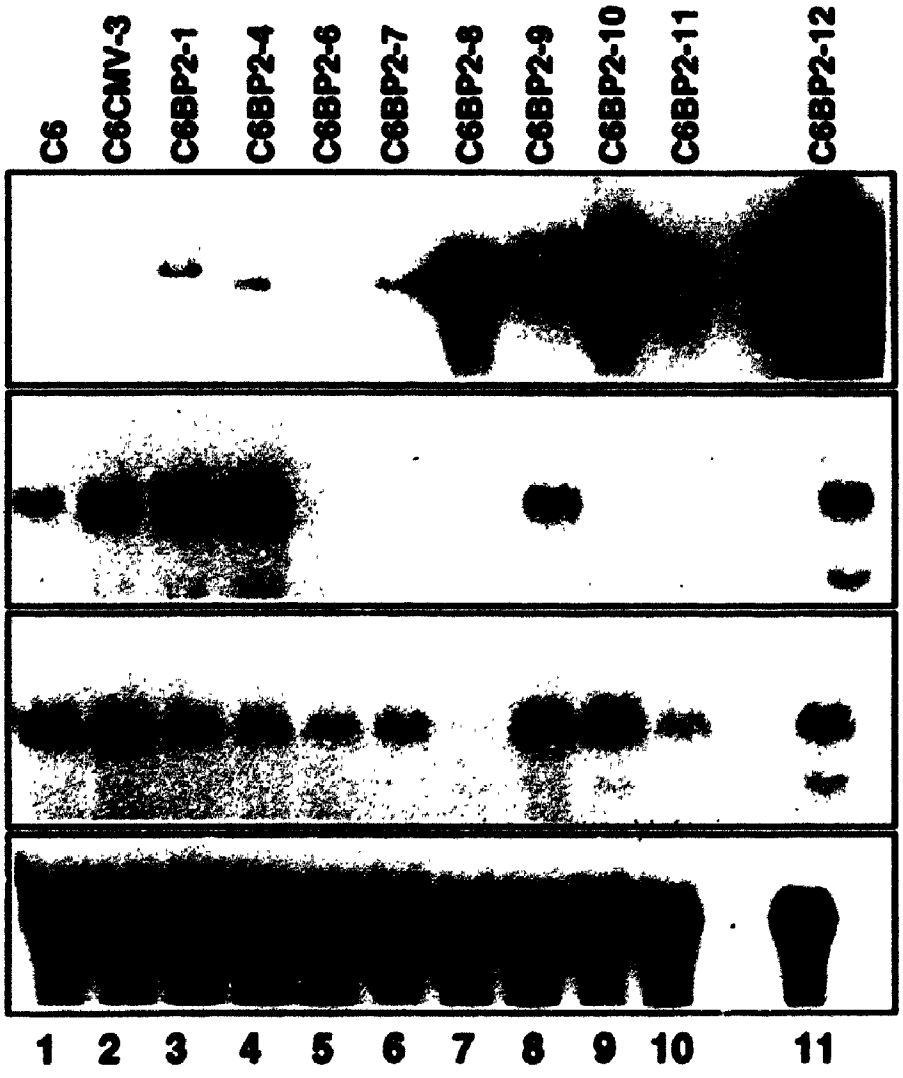
Nine G418 resistant clones were selected and the conditioned media, as analyzed by ligand blotting, showed variable levels of a 34 kDa IGFBP (IGFBP-2). Clones C6BP2- 8, -10, -11 and -12 secreted relatively high levels of this protein, while clones C6BP2-1, -4, -6, -7 and -9 secreted lower levels. The identity of this protein was confirmed by immunoblotting with a specific antiserum against bovine IGFBP-2 (Figure 6.1 B). The levels of immunoreactive IGFBP-2 corresponded to the levels of IGFBP-2 observed by ligand blotting. The vector transfected control clone (C6CMV-3) did not express IGFBP-2 and was chosen as an appropriate control over other vector control clones as the IGFBP and IGF-I expression profile was most similar to the wild type C6 cells.

The levels of the other IGFBPs in the various clones were also variably altered. IGFBP-3 was downregulated, to a variable degree, in all clones. Clones C6BP2-7 and -9 had just detectable levels of IGFBP-3 protein, while clones C6BP2-8, -10, and -11 lost all detectable levels. IGFBP-4 was also downregulated in all clones except for the vector control C6CMV-3. The 28 kDa IGFBP was detected in only clones C6CMV-3 and C6BP2-10. Clones C6BP2-1, -4, -7, and -9 expressed an additional IGFBP of 29 kDa.

6.4.2 Expression of the IGFBP genes

Expression of the transfected ovine IGFBP-2 cDNA was analyzed by Northern blotting to determine the steady state mRNA levels (Figure 6.2).

Figure 6.2 Northern blot analysis of total RNAs (20 μg/lane) from C6 glioma (lane 1), pRc/CMV vector transfected control (lane 2), and pRc/CMV-oIGFBP-2 transfected clones (lanes 3-11) sequentially probed with [^{32}P]labeled cDNAs for IGFBP-2 (first panel), IGFBP-3 (second panel), IGFBP-4 (third panel) and 18S ribosomal RNA (fourth panel). A 1.6 kb IGFBP-2 transcript was readily detectable only in the pRc/CMV-oIGFBP-2 transfected clones. The 2.6 kb IGFBP-3 transcript and the 2.4 kb IGFBP-4 transcript were observed in various clones. Relative consistency in loading and transfer of total RNA is shown in the fourth panel by hybridization to 18S ribosomal RNA. IGFBP-1, IGFBP-5 and IGFBP-6 mRNAs were not detected (not shown).


C6
C6CMV-3
C6BP2-1
C6BP2-4
C6BP2-6
C6BP2-7
C6BP2-8
C6BP2-9
C6BP2-10
C6BP2-11
C6BP2-12
1 2 3 4 5 6 7 8 9 10 11

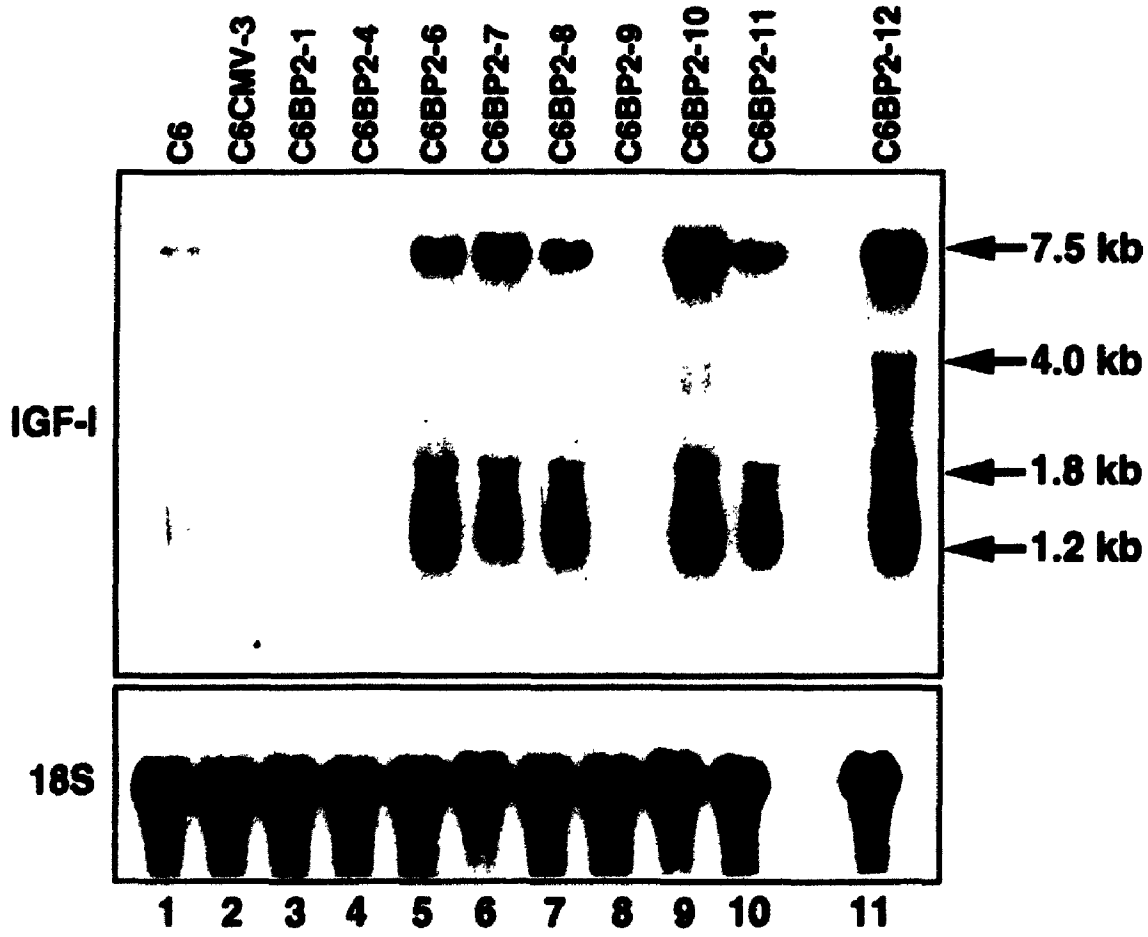
IGFBP-2 mRNA was not detected in the wild type C6 glioma cells, nor the vector control clone C6CMV-3. A 1.6 kb transcript was detected, at variable levels, in the IGFBP-2 secreting clones. The relative levels of IGFBP-2 steady state mRNAs corresponded to the relative amount of secreted IGFBP-2 protein for each clone. Clones C6BP2-8, -10, -11 and -12 expressed relatively high levels of IGFBP-2 mRNA while clones C6BP2-1, -4, -6, -7 and -9 expressed lower levels of IGFBP-2 mRNA.

A 2.6 kb IGFBP-3 and 2.4 kb IGFBP-4 transcripts were also detected by Northern blotting (Figure 6.2). The relative amount of IGFBP-3 steady state mRNA detected for each clone did not necessarily correspond to the amount of secreted IGFBP-3 protein, suggesting that changes in post-transcriptional regulation of the IGFBP-3 gene might have occurred. The relative levels of IGFBP-4 steady state mRNA for each clone corresponded to the levels of secreted IGFBP-4 protein. IGFBP-1, -5, and -6 mRNAs were not detected (not shown).

6.4.3 Expression of the IGF-I gene

IGF-I stable mRNA levels were examined in the wild type C6 glioma cells and the various clones by Northern analysis (Figure 6.3). A major 7.5 kb transcript was detected in addition to minor transcripts of 4.0, 2.0 and 1.2 kb. The relative level of IGF-I steady state mRNA was low in the wild type C6 cells. Clones C6BP2-1, -4, -9, and C6CMV-3 had similarly low levels of IGF-I mRNA as compared to the wild type C6 cells. IGF-I steady state mRNA was upregulated in clones C6BP2-6, -7, -8, -10 and -12 as compared to the wild type C6 cells. The 7.5 and 1.2 kb IGF-I transcripts were preferentially upregulated in these clones. IGF-II mRNA was not detected (not shown).

Figure 6.3 Northern blot analysis of total RNAs (20 μg/lane) from C6 glioma (lane 1), pRc/CMV vector transfected control (lane 2), and pRc/CMV-oIGFBP-2 transfected clones (lanes 3-11) probed with [^{32}P]labeled cDNAs for rat IGF-I (upper panel) and 18 S ribosomal RNA (lower panel). The clones expressed variable levels of four distinct IGF-I transcripts of 7.5, 4.0, 1.8 and 1.2 kb. IGF-II mRNA was not detected (not shown).


C6
C6CMV-3
C6BP2-1
C6BP2-4
C6BP2-6
C6BP2-7
C6BP2-8
C6BP2-9
C6BP2-10
C6BP2-11
C6BP2-12
IGF-I
7.5 kb
4.0 kb
1.8 kb
1.2 kb
18S
1 2 3 4 5 6 7 8 9 10 11

The levels of radioimmunoassayable IGF-I measured in the CM from the wild type C6 cells ranged from 0.496-4.18 ng/ml, amounts similar to that previously reported by others (Kiess et al. 1989). Clones C6BP2-1, -4, -6, -7 and -9 secreted lower levels of IGF-I compared to the wild type C6 cells, while clones C6BP2-8. -10, -11 and -12 all secreted higher amounts of IGF-I (Table 6.1). In most clones, the relative level of secreted IGF-I corresponded to the relative level of secreted IGFBP-2. Clones C6BP2-8 and -12 which secreted the highest levels of IGFBP-2, also secreted the highest levels of IGF-I.

6.4.4 Analysis of the growth rates of the C6 clones transfected with an IGFBP-2 cDNA

To determine the effect of IGFBP-2 overexpression on glial cell growth, the growth rates of selected clones were determined. Clones C6BP2-10 and -12 were chosen for their high level IGFBP-2 expression, while clones C6BP2-1, -4 and -9 were chosen for their moderate expression of IGFBP-2. Growth curves were constructed by counting cell number over a period of 72 h. Representative growth curves for C6 cells and clones C6BP2-1 and -12 are shown in Figure 6.4 A. C6BP2-12 which highly overexpressed the IGFBP-2 cDNA, exhibited a similar increase in cell number over 72 h, compared to the wild type C6 cells. In contrast, the moderate expressor of IGFBP-2, clone C6BP2-1, had a significant reduction in cell number at 48 and 72 h, when compared to the wild type C6 cells.

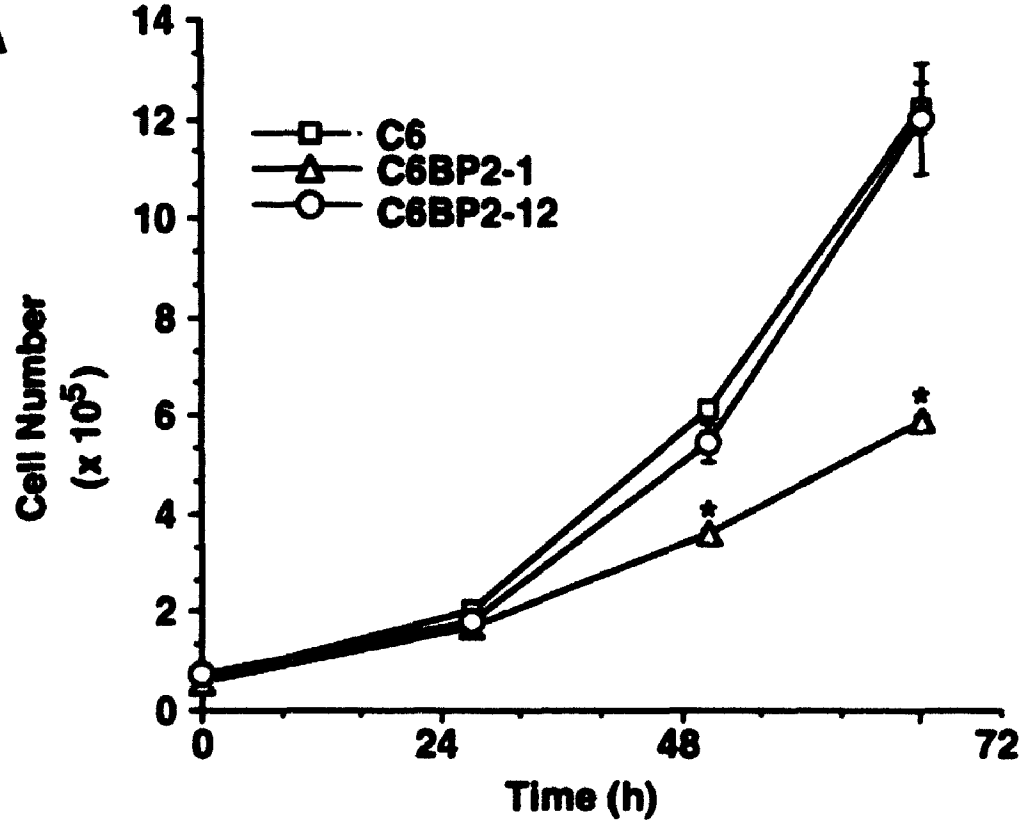
The doubling time for each clone was calculated from three separate experiments and the mean ± SEM calculated (Figure 6.4 B). Clones C6CMV-3, C6BP2-10 and -12 had similar doubling times compared to the wild type C6 cells. In contrast, the moderate expressors of IGFBP-2, clones C6BP2-1, -4 and -9, had significantly greater doubling times compared to the C6 cells. Of

Table 6.1 The concentration of IGF-I in 24 h conditioned media of C6 (wild type), CMV-3 (vector control), and C6 IGFBP-2 transfected clones determined by radioimmunoassay. Values are the mean ± SEM of three separate collections from each clone.

Cell Type	IGF-I (ng/ml)
C6	2.29 ± 1.06
C6CMV-3	2.09 ± 1.92
C6BP2-1	0.26 ± 0.14
C6BP2-4	0.81 ± 0.50
C6BP2-6	1.48 ± 0.72
C6BP2-7	0.72 ± 0.05
C6BP2-8	6.36 ± 2.46
C6BP2-9	0.49 ± 0.35
C6BP2-10	3.03 ± 2.36
C6BP2-11	5.12 ± 3.24
C6BP2-12	13.54 ± 4.96

Figure 6.4 (A) Growth curves were constructed by plotting the mean cell number against time over a period of 72 hours for the wild type C6 cells (●), C6BP2-1 (▲) and C6BP2-12 (■). The wild type C6 cells demonstrated an exponential growth rate. C6BP2-12 exhibited a similar growth rate compared to C6 cells. C6BP2-1 exhibited a reduced growth rate compared to C6 cells. The cell number for C6BP2-1 was significantly less than that for C6 cells at 48 and 72 h, *, statistically significant change in cell number when compared to C6 cells, $p<0.05$. (B) The doubling times were calculated from the growth curves for each clone. The mean $\pm$ SEM are shown from three separate experiments for each clone indicated. * indicates statistically significant difference when compared to C6 cells, $p<0.05$.

A



B

Cell Type	Doubling Time (hours) mean ± SEM
C6	15.3 ± 0.7
C6CMV-3	16.0 ± 0.8
C6BP2-1	19.1 ± 0.8 *
C6BP2-4	19.5 ± 1.8 *
C6BP2-9	19.4 ± 2.1 *
C6BP2-10	15.7 ± 0.8
C6BP2-12	16.2 ± 1.0

significance, clones C6BP2-1, -4 and -9 also expressed very low levels of IGF-I mRNA and peptide, while clones C6BP2-10 and -12 expressed high levels of IGF-I (Figure 6.3 and Table 6.1).

6.5 DISCUSSION

6.5.1 Expression of IGFBPs and their relationship to growth in the C6 clones transfected with an IGFBP-2 cDNA

The ovine IGFBP-2 cDNA was overexpressed in C6 glioma cells to address the biologic role of endogenous IGFBP-2 in astroglial cell growth. This study was based on the observation that cells with regulated growth (primary astroglia) express abundant IGFBP-2, whereas those with unregulated growth (glioma cells) express very little IGFBP-2 (Chapter 5). To study cells with varying capacities to synthesize IGFBP-2, several clones were selected based on the level of expression of the IGFBP-2 cDNA. We found that changes in the growth rate of the clones from the wild type C6 cells did not correspond to the level of over-expression of the IGFBP-2 cDNA. This finding was unexpected since we have previously demonstrated that exogenous IGFBP-2 inhibits IGF stimulated [^{3}H]thymidine incorporation of primary astroglial cells (Han et al. 1988b). To our surprise, we noted that clones overexpressing very high levels of the IGFBP-2 cDNA, also expressed correspondingly high levels of IGF-I mRNA and peptide, and had similar growth rates compared to the wild type C6 cells. In contrast, clones overexpressing moderate levels of the IGFBP-2 cDNA, with no increase in IGF-I mRNA and peptide had significantly reduced growth rates compared to

the wild type C6 cells. These findings suggest that growth of C6 cells may be closely linked to their capacity to regulate the synthesis of IGF-I.

Not only was the level of IGFBP-2 synthesis by the various clones different, but other endogenous C6 IGFBPs were variably altered. IGFBP-3 was downregulated, and IGFBP-4 and the 28 kDa IGFBP were either secreted at wild type levels or downregulated. These findings suggest that C6 glioma cell growth is not regulated by IGFBP-3 or IGFBP-4. Interestingly, a 29 kDa IGFBP, not found in the wild type cells, was observed in some of the transfected clones. The mechanism for the changes in endogenous IGFBP expression was not examined however, variation in the site of integration of the cDNA may have affected regulatory elements controlling endogenous IGFBP expression. Differences between steady state mRNA levels and amount of protein detected in the conditioned medium suggest that post-transcriptional and/or post-translational processing may have occurred. Specific protease activity has been described for IGFBP-3 during pregnancy (Hossenlopp et al. 1990, Guidice et al. 1990, Davenport et al. 1992a) and other catabolic states (Davies et al. 1991), for IGFBP-4 in neural cells (Fowlkes et al. 1992), and for IGFBP-5 (Nam et al. 1994, Thrailkill et al. 1995). Differences between steady state mRNA levels and amount of protein detected in the conditioned medium could also be accounted for by the presence of cell surface associated IGFBPs. Cell surface association for IGFBP-1, IGFBP-2 and IGFBP-3 has been described in a number of different *in vitro* systems (Jones et al. 1993b, Delhanty and Han 1993, Reeve et al. 1993, Oh et al. 1993b).

Clones which overexpressed a greater amount of the IGFBP-2 cDNA tended to downregulate the other IGFBPs. Mice in which the IGFBP-2 gene has been disrupted by gene targeting, showed an upregulation of serum IGFBP-3 and IGFBP-4 (Wood et al. 1993). These changes in IGFBP expression

in response to a perturbation in endogenous IGFBPs may be a compensatory response designed to maintain an appropriate balance of growth promoting and growth inhibiting IGFBPs. These changes in endogenous IGFBPs, seen both in the IGFBP-2 null mice and the clones which highly overexpressed the IGFBP-2 cDNA, may account for the lack of phenotype in the IGFBP-2 null mice and the lack of growth changes observed in some of the clones overexpressing the IGFBP-2 cDNA.

6.5.2 Expression of IGF-I and its relationship to growth in the C6 clones transfected with an IGFBP-2 cDNA

Upon analysis of the growth rates of selected clones, we were able to divide the clones into two separate groups, those clones that grew at rates similar to the wild type cells and those clones that grew about 30% slower than the wild type cells. Interestingly, the clones that had very high levels of expression of the IGFBP-2 cDNA grew at the same rate as the wild type cells, while the clones that moderately overexpressed IGFBP-2 grew at slower rates. When the IGF-I levels were examined, the slow growing clones had reduced levels of IGF-I compared to the wild type cells, while the fast growing clones had IGF-I mRNA levels similar to or greater than the wild type cells. IGF-I has been shown to be an important regulator of C6 cell growth. Reduction of endogenous IGF-I production in C6 cells by transfection of an antisense cDNA, resulted in the loss of tumourigenicity of C6 cells *in vivo* (Trojan et al. 1992). Similar studies in other cell types have demonstrated that loss of expression of IGF-I leads to reduced growth and tumourigenicity (Pietrzkowski et al. 1992b, Ambrose et al. 1994). We have previously shown that when C6 cells are transfected with the gap junction cDNA connexin43, the reduction in growth rate of the clones overexpressing the connexin43

cDNA is also associated with a reduction in the level of expression of the IGF-I gene (Chapter 5). Our results support the hypothesis that the interaction of IGF-I with its receptor is an important regulator of C6 cell growth.

Those clones that highly overexpressed IGFBP-2 had compensatory upregulation of IGF-I which may account for their maintenance of a wild type growth rate. The mechanism for this upregulation in IGF-I expression is unclear. The rat IGF-I gene is characterized by multiple transcription initiation sites. Multiple promoter elements and some repressor elements have also been described for this gene, however, very little is known about the possible *trans*-acting factors that regulate the expression of this gene. It has been determined that the rat IGF-I gene contains an E2F binding sequence which acts as a repressor. Proteins that were identified to interact with this sequence and participate in the negative control of the IGF-I gene were, E2F, cyclin A, and the retinoblastoma gene product, p107. The Simian Virus 40 large T antigen was able to disaggregate these proteins to activate transcription of the IGF-I gene, suggesting that activation of the IGF-I gene is closely linked to regulators of the cell cycle (Porcu et al. 1994). Whether the upregulation of IGF-I seen in the IGFBP-2 transfected clones is a result of perturbations in cell cycle regulators, or the result of random integration remains to be determined. Irrespective of the reason for upregulation of IGF-I gene expression, it is important to note that this compensatory affect is associated with a maintenance of normal (wild type) growth rate, whereas failure to do so results in impaired growth.

6.5.3 Other factors responsible for the changes in growth

A decrease of IGF-I receptors, or a reduction in IGF affinity, may also explain the reduction in growth of clones C6BP2-1, -4, and -9. Monolayer

growth was inhibited, and tumourigenicity was lost both *in vitro* and *in vivo*. when expression of the IGF-I receptor was blocked by antisense oligonucleotides or cDNA in C6 cells (Resnicoff et al. 1994a). Similar studies in other cell types have demonstrated that loss of expression of the IGF-I receptor leads to reduced growth and tumourigenicity (Pietrzkowski et al. 1992b, Ambrose et al. 1994, Resnicoff et al. 1994b).

Changes in expression of other endogenous growth factors may also account for the differences in growth between the various clones. Also, changes in expression of endogenous growth factors may have resulted from the effects of random integration or be directly regulated by components of the IGF system.

6.6 CONCLUSION

The exact mechanism(s) controlling the growth of the various C6BP2 clones remains to be determined, however, it is clear that the interactions between IGF-I and IGFBPs remain an important determinant of their growth. Whether IGFBP-2 plays an unique role in the growth of glial cells remains to be clarified. This study suggests that glial cells may have a limited capacity to modulate various components of the IGF system in order to maintain normal growth as evidenced by the concomitant upregulation of IGF-I. These studies suggest that growth of glial cells is regulated by a coordinated interaction between IGF-I and IGFBPs.

CHAPTER SEVEN

DETECTION AND CHARACTERIZATION OF A CELL SURFACE ASSOCIATED IGFBP FOUND ON THE SLOW GROWING CLONES.

7.1 INTRODUCTION

In the previous chapter, the characterization of nine clones that had been transfected with an ovine IGFBP-2 cDNA was described and the growth characteristics of a selected few were analyzed. Clones C6BP2-10 and -12 grew at a similar rate to the wild type C6 cells and the vector control clone C6CMV-3. Clones C6BP2-1, -4, and -9 grew at significantly reduced rates compared to the wild type cells. The fast growing clones upregulated their expression of the IGF-I gene which may account for their maintenance of a wild type growth rate. Alternatively, the clones may exhibit differences in receptor number or affinity which could explain the different growth phenotypes.

Affinity cross-linking with [^{125}I]IGF-I and [^{125}I]IGF-II revealed a cell surface associated protein that bound IGFs and had a molecular weight in the range of IGFBPs. Cell surface association of IGFBPs has been described for IGFBP-1, IGFBP-2, IGFBP-3 and IGFBP-5 (Jones et al. 1993b, Reeve et al. 1993, Delhanty and Han 1993, Conover et al. 1990, Oh et al. 1993b, Reeve et al. 1995). Both IGFBP-1 and IGFBP-2 have RGD integrin recognition motifs in their carboxyl terminal region. IGFBP-1 associates with the cell surface via its RGD sequence by interaction with the $\alpha_5\beta_1$ integrin (Jones et al. 1993b). Whether IGFBP-2 associates with the cell surface via its RGD motif has not been demonstrated. Both IGFBP-3 and IGFBP-5 contain heparin binding motifs through which they may bind to heparin molecules on the surface of cell membranes. Heparin has been shown to inhibit the cell surface association of IGFBP-3 and IGFBP-5 (Booth et al. 1994). Other studies have demonstrated that IGFBP-3 is associated with specific proteins on the surface membrane of breast cancer cells (Oh et al. 1993b). Cell surface association of IGFBP-2, IGFBP-3 and IGFBP-5 have been associated with changes in cell growth suggesting

that the cell surface associated IGFBP observed on some of the IGFBP-2 transfected C6 clones may be related to the observed differences in growth rates (Reeve et al. 1993, Oh et al. 1993b, Reeve et al. 1995).

7.2 OBJECTIVES

The objectives of this study were to examine the IGF-I and IGF-II receptor binding affinity in clones C6BP2-1, -4, and -9 as possible contributors to the slow growth phenotype, and secondly, to characterize the membrane associated IGF3P identified by affinity cross-linking and determine whether it may contribute to the observed changes in growth rate.

7.3 METHODS

7.3.1 Affinity cross-linking

To examine the receptor binding characteristics, [^{125}I]IGF-I or [^{125}I]IGF-II were cross-linked to monolayers of the selected clones, in the absence or presence of variable concentrations of unlabeled IGF-I, IGF-II and insulin as described in section 2.7. Heparin and the hexapeptide GRGDSP were used to examine the binding competition characteristics of the cell surface associated IGFBP.

7.3.2 Immunoprecipitation

[^{125}-I]IGF-II-IGFBP complexes from conditioned media and cell monolayers of C6, clones C6BP2-1, -4, and -12 were immunoprecipitated with an antiserum against bovine IGFBP-2 as described in section 2.8.

7.3.3 Ligand blot analysis

Conditioned media was collected from FRTL-5 cells grown in Coon's F-12 medium with 5% newborn calf serum, supplemented with TSH (1 mU/ml), insulin (10µg/ml), and transferrin (5 µg/ml) and used as a positive control for IGFBP-5 (a generous gift of Dr. G. Becks, Lawson Research Institute, London, Ont.). Ligand blot analysis was performed as described in section 2.3 using conditioned media from clones C6BP2-1, -4, and -12 and rRTL-5 cells.

7.3.4 Northern blot analysis

Total RNA was extracted from 0.5 g each of adult rat liver and kidney as described in section 2.5.1 and used as positive controls in Northern blot analysis. Total RNA (40 µg/lane) from C6, the vector control C6CMV-3, and the IGFBP-2 transfected clones was re-analyzed by Northern blotting as described in section 2.5. The blots were hybridized with labeled cDNAs for rat IGFBP-5 and 18 S ribosomal RNA.

7.3.5 Growth studies

Growth of C6 cells and clones C6BP2-1 and -12 in 1% FBS with or without the addition of exogenous IGF-I was analyzed as described in section 2.10. Cells were plated at a density of 2 x 10^5 cells in 6 well tissue culture plates in DMEM with 10% FBS. After 24 h, the cells were rinsed and placed in

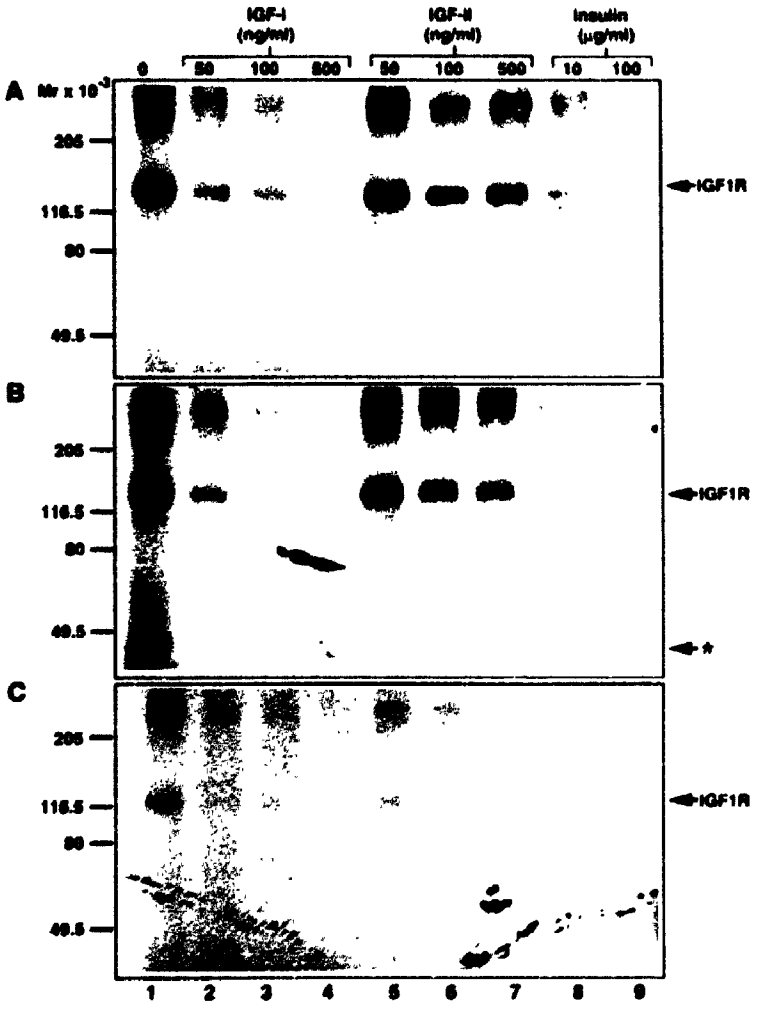
medium containing 1% FBS with or without the daily addition of 200 ng/ml of IGF-I. Triplicate wells were counted using a Coulter counter® at this time zero and every 24 h thereafter, for a period of 72 h. Mean cell number and SEM were calculated and changes in cell number with or without IGF-I treatment were compared within each clone using analysis of variance.

7.4 RESULTS

7.4.1 Affinity cross-linking of IGFs

Cross-linking of [^{125}I]IGF-I or [^{125}-I]IGF-II, to monolayers of selected clones, in the absence or presence of variable concentrations of unlabeled IGF-I, IGF-II and insulin, was used to examine relative IGF receptor binding affinity. When IGF-I was used as the radioligand, all clones examined displayed a 135 kDa band consistent in size with the IGF-I receptor α-subunit, in addition to, a 270 kDa band consistent in size with α-subunit dimers (Figure 7.1 A-C). Binding of [^{125}I]IGF-I was competed with unlabeled IGF-I, to a lesser degree with IGF-II, and with very high concentrations of insulin (lanes 8, 9). This characteristic binding pattern of the IGF-I receptor was observed with wild type C6 cells (A) and all clones examined, C6BP2-1 (B) and C6BP2-12 (C), (C6CMV-3, C6BP2-4, -9 and -10, data not shown). An additional band of 41 kDa was observed in clones C6BP2-1 (Figure 7.1 B), -4 and -9 (not shown). This band was competed with excess IGF-I and IGF-II, but not with insulin, suggesting that it was a membrane associated IGFBP. The IGFs competed more effectively for binding to the 41 kDa membrane associated IGFBP than the IGF-I receptor suggesting that the membrane associated IGFBP had a greater affinity for IGF-I compared to the IGF-I receptor.

Figure 7.1 Affinity cross-linking of [^{125}I]IGF-I to monolayers of wild type C6 (**A**), clone C6BP2-1 (**B**) and clone C6BP2-12 (**C**). All clones displayed a 135 kDa type I receptor α-subunit (IGF1R) monomer and 270 kDa α-subunit dimer. Binding was competed by addition of exogenous IGF-I (2-4), IGF-II (lanes 5-7) to a lesser degree, and by very high concentrations of insulin (lanes 8-9). The competition of [^{125}I]IGF-I was similar in all clones examined (C6CMV-3, C6BP2-4, -9, and -10, data not shown). Clone C6BP2-1 displayed an additional band of 41 kDa (•) that was competed by the addition of IGF-I and IGF-II but not insulin (**B**). The 41 kDa band was also observed with clones C6BP2-4 and -9 (data not shown).


IGF-I (ng/ml)
IGF-II (ng/ml)
Insulin (µg/ml)
0
50
100
500
50
100
500
10
100
A
B
C
Mr x 10⁻³
205
116.5
80
49.5
IGF1R
*
1
2
3
4
5
6
7
8
9

When [^{125}I]IGF-II was used as the radioligand all clones displayed a 260 kDa band consistent in size with the IGF-II receptor (Figure 7.2 A-C). This band was competed most effectively by unlabeled IGF-II, to a lesser degree by IGF-I, and not at all by insulin, consistent with the competition profile of the IGF-II receptor. The binding competition profile of [^{125}I]IGF-II was similar in the wild type C6 cells and in all clones examined (C6CMV-3, C6BP2-4, -9, and -10, not shown). The 41 kDa band was also observed in clones C6BP2-1 (Figure 7.2 B), C6BP2-4 and -9 (data not shown) when [^{125}I]IGF-II was used as radioligand. Its binding was also competed by unlabeled IGF-I and IGF-II, but not insulin, again suggesting its identity as a membrane associated IGFBP. The IGFs competed more effectively for binding to the 41 kDa membrane associated IGFBP than the IGF-II receptor again suggesting that it had a greater affinity for IGF-II than the IGF-II receptor.

7.4.2 Immunoprecipitation of [^{125}I]IGF-II-IGFBP-2 cross-linked complexes

An antiserum against bovine IGFBP-2 was used to immunoprecipitate [^{125}I]IGF-II cross-linked proteins from conditioned media and cell monolayers of selected clones (Figure 7.3). In the conditioned media of clone C6BP2-12, a large amount of [^{125}I]IGF-II-IGFBP-2 cross-linked complexes were immunoprecipitated whereas, a lesser amount from clones C6BP2-1 and -4, and a very small amount from C6 were immunoprecipitated. The size of the [^{125}I]IGF-II-IGFBP-2 immunoprecipitated complex was slightly larger than the [^{125}I]IGF-II-IGFBP cross-linked complex found on the cell surface of C6BP2-1.

The IGFBP-2 antiserum immunoprecipitated an additional [^{125}I]IGF-II-IGFBP complex of smaller size. This was detected in clones C6BP2-1 and -4, both in the conditioned media and on the cell surface. This [^{125}I]IGF-II-IGFBP complex migrated at the same position as the membrane associated IGFBP

Figure 7.2 [^{125}I]IGF-II was used to affinity cross-link monolayers of wild type C6 (**A**), clone C6BP2-1 (**B**) and clone C6BP2-12 (**C**). All clones displayed a band of 260 kDa consistant with the type II receptor (IGF2R). Binding was competed most effectively by the addition of IGF-II (lanes 2-4), to a lesser degree by IGF-I (lanes 5-7) and not at all by insulin (lanes 8-9). The competition of [^{125}I]IGF-II was similar in all clones examined (C6CMV-3, C6BP2-4, -9 and -10, data not shown). The 41 kDa band (*) was also observed in clones C6BP2-1 (**B**), C6BP2-4 and -9 (data not shown) when IGF-II was used as radioligand. Competition was observed with the addition of IGF-II and IGF-I but not insulin.

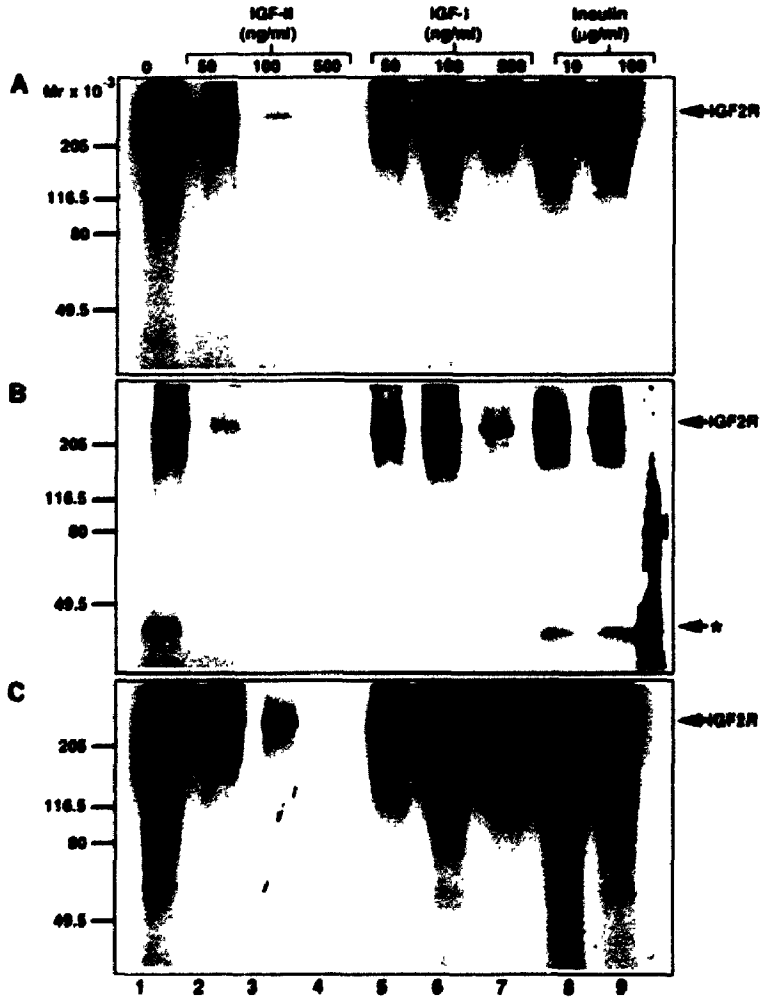
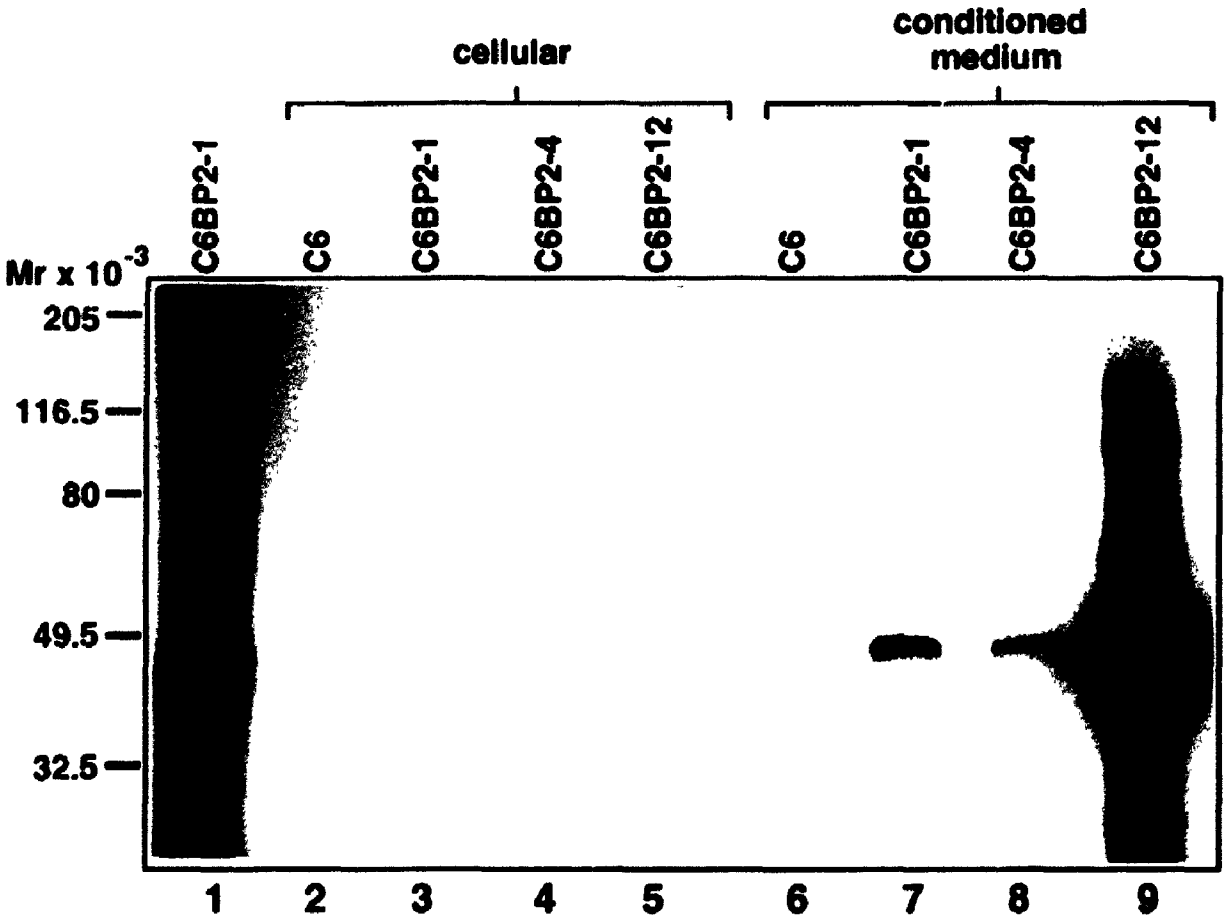

IGF-II (ng/ml)
IGF-I (ng/ml)
Insulin (μg/ml)
0
50
100
500
50
100
500
10
100
A
Mr x 10⁻³
205
116.5
80
49.5
IGF2R
B
205
116.5
80
49.5
IGF2R
*
C
205
116.5
80
49.5
IGF2R
1
2
3
4
5
6
7
8
9

Figure 7.3. [^{125}I]IGF-II was affinity cross-linked to both monolayer cultures and conditioned medium from C6, clones C6BP2-1, -4, and -12, immunoprecipitated with antiserum against bovine IGFBP-2, subjected to SDS-PAGE, and immune complexes were visualized by autoradiography. Immunoreactive [^{125}I]IGF-II-IGFBP complex of 44 kDa was observed in the CM of C6, and clones C6BP2-1, -4, and -12. An additional [^{125}I]IGF-II-IGFBP complex of 41 kDa was immunoprecipitated from CM and cell monolayers of clones C6BP2-1 and -4 which migrated at the same relative molecular weight as the [^{125}I]IGF-II crosslinked species from clone C6BP2-1. Lane 1, C6BP2-1 [^{125}I]IGF-II cross-linked only; lanes 2-5, immunoprecipitated [^{125}I]IGF-II-IGFBP complexes from cell monolayers of C6, clones C6BP2-1, -4, and -12; lanes 6-9, immunoprecipitated [^{125}I]IGF-II-IGFBP complexes from CM of C6, clones C6BP2-1, -4, and -12.


cellular
conditioned medium
C6BP2-1
C6
C6BP2-1
C6BP2-4
C6BP2-12
C6
C6BP2-1
C6BP2-4
C6BP2-12
Mr x 10-3
205
116.5
80
49.5
32.5
1
2
3
4
5
6
7
8
9

found on clone C6BP2-1, suggesting that the identity of the membrane associated IGFBP was not IGFBP-2, but rather an IGFBP that crossreacted with the IGFBP-2 antiserum.

7.4.3 Competition of binding of the cell surface associated IGFBP

IGFBP-2 contains an Arg-Gly-Asp (RGD) integrin recognition motif in its carboxyl terminal end. Delhanty and Han (1993) have shown that IGFBP-2 will associate to the cell surface of sheep choroid plexus cells via interaction with its RGD domain and mutation of the aspartate to glutamate will block this interaction. IGFBP-1 also has an RGD integrin recognition motif and has been shown to associate with the cell surface of fibroblasts via this motif (Jones et al. 1993b). Peptides containing the RGD integrin recognition motif were used in the affinity cross-linking assay to determine whether the cell surface associated IGFBP might be interacting via an RGD domain. The hexapeptide GRGDSP did not inhibit the cross-linking of [^{125}I]IGF-II to the cell surface associated IGFBP (Figure 7.4) further suggesting that the cell surface associated IGFBP was not IGFBP-2.

A putative heparin binding domain has been identified in IGFBP-5 and has been shown to be important in the association of IGFBP-5 with the cell surface of endothelial cells (Booth et al. 1994). Heparin was used in the affinity cross-linking assay to determine whether the cell surface associated IGFBP might be interacting via a heparin binding domain. Cross-linking of the [^{125}I]IGF-I (Figure 7.5) or [^{125}I]IGF-II (not shown) to the cell surface IGFBP of clone C6BP2-1 was reduced to a small degree by the addition of very high concentrations of heparin.

Figure 7.4 [^{125}I]IGF-II was affinity crosslinked to C6 cells and clone C6BP2-1 without or with the peptide containing the integrin recognition motif RGD (GRGDSP). Lane 1: C6 cells, lane 2: clone C6BP2-1 alone, lane 3: clone C6BP2-1 with 100 ng/ml GRGDSP, lane 4: clone C6BP2-1 with 1000 ng/ml GRGDSP. The 240 kDa IGF2R was observed in addition to the 41 kDa membrane associated IGFBP. The RGD containing peptide did not inhibit the membrane association of the IGFBP.

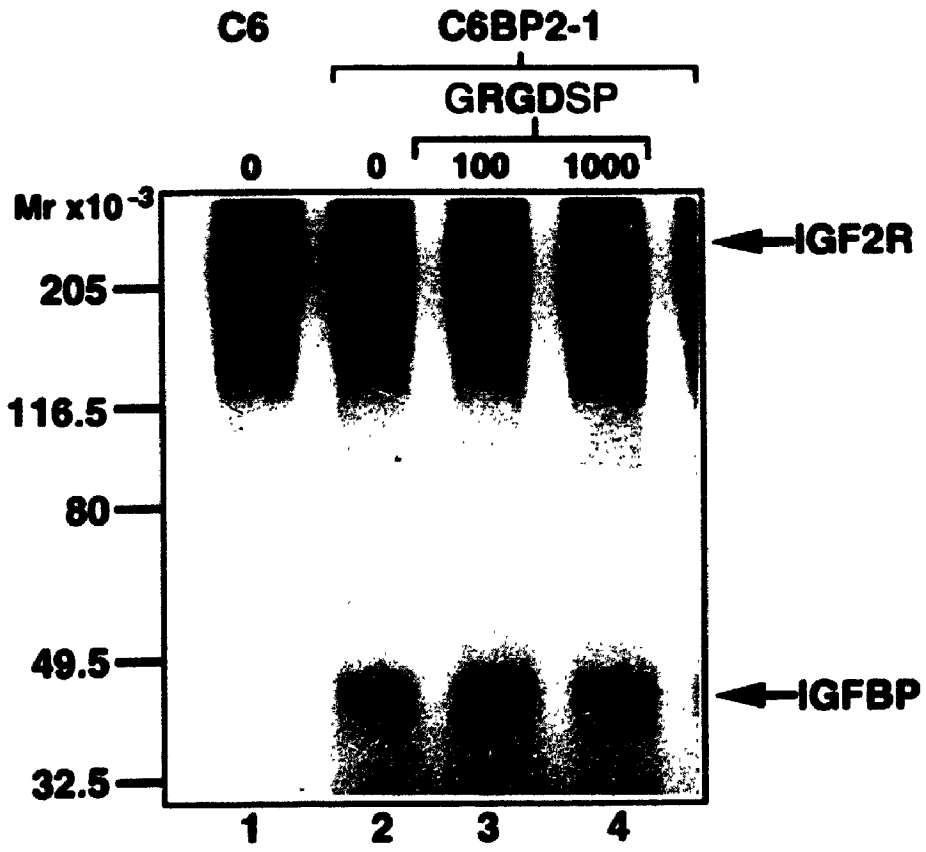
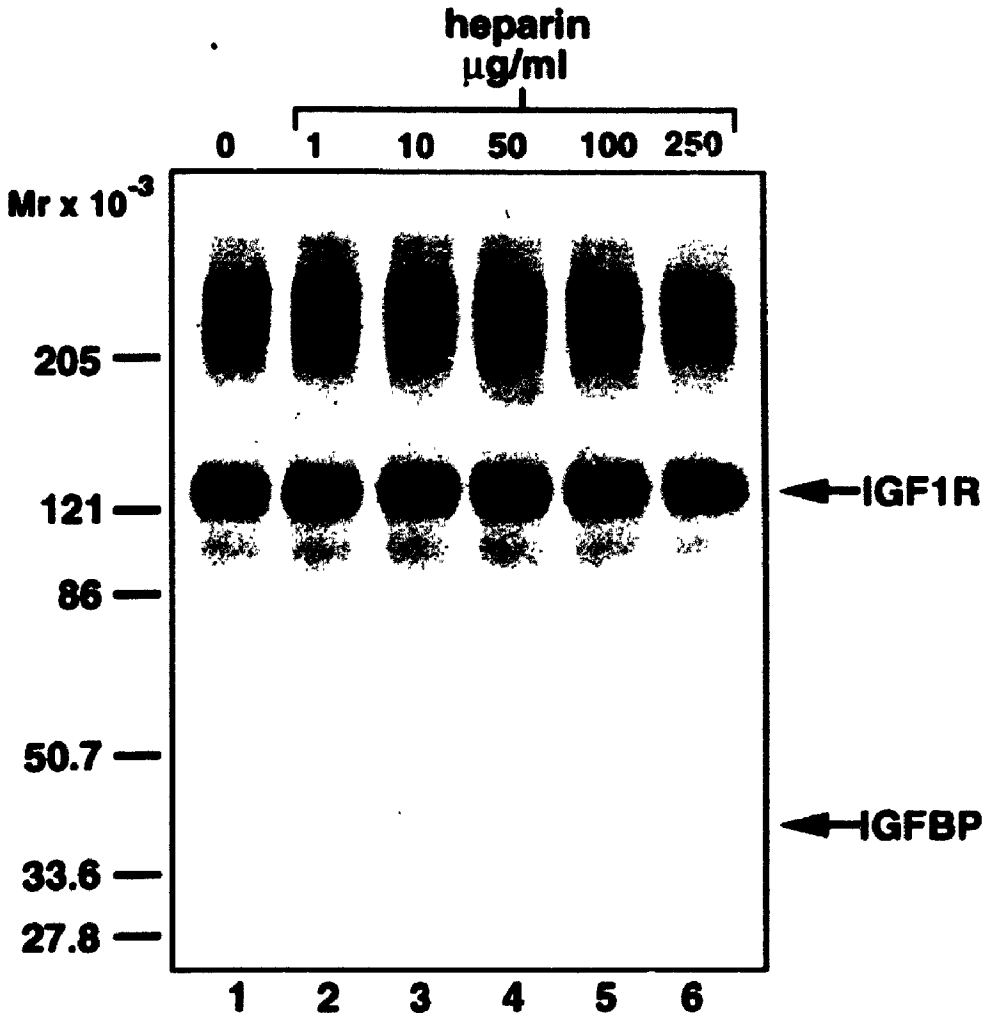

C6
C6BP2-1
GRGDSP
0
0
100
1000
Mr x10-3
205
116.5
80
49.5
32.5
IGF2R
IGFBP
1
2
3
4

Figure 7.5 [^{125}I]IGF-I was affinity crosslinked to clone C6BP2-1 without (lane 1) or with (lanes 2-6) increasing concentrations of heparin (1-250 μg/ml). The binding of [^{125}I]IGF-I to the 41 kDa cell surface associated IGFBP was competed slightly with very high concentrations of heparin. The binding of [^{125}I]IGF-I to the type 1 receptor α-subunit was not affected.


heparin
µg/ml
0
1
10
50
100
250
Mr x 10-3
205
121
86
50.7
33.6
27.8
IGF1R
IGFBP
1
2
3
4
5
6

7.4.4 The 29 kDa IGFBP co-migrates with IGFBP-5

The rat thyroid cell line FRTL-5 secretes IGFBP-5 as its predominant IGFBP in the conditioned media (Backeljauw et al. 1993). Ligand blot analysis was used to determine whether the 29 kDa IGFBP found in the conditioned media of clones C6BP2-1, -4, and -9 co-migrated with the characterized IGFBP-5 of FRTL-5 cells. Figure 7.6 demonstrates that FRTL-5 cells secrete a 29 kDa IGFBP-5 and a 22 kDa proteolytic fragment of IGFBP-5 (Backeljauw et al. 1993). This characterized IGFBP-5 co-migrates with the 29 kDa IGFBP found in the conditioned media of clones C6BP2-1, -4, and -9, but not clone C6BP2-12. Three different antiserum for IGFBP-5 were used in Western blotting and were unable to recognize IGFBP-5 in the CM of FRTL-5 cells.

7.4.5 Detection of IGFBP-5 mRNA transcripts

Total RNA (40 µg/lane) from the different clones was re-examined by Northern blotting for the expression of IGFBP-1, -5, and -6 mRNAs. A 6.0 kb IGFBP-5 transcript was detected in rat kidney and just detectable in clones C6BP2-1 and -4 (Figure 7.7). When the same blot was re-probed for IGFBP-1 or IGFBP-6, mRNA transcripts of neither IGFBP were detected in any of the transfected clones (not shown). This data, along with the relative molecular weight and co-migration with IGFBP-5, suggests that the 29 kDa IGFBP found in the conditioned media and associated with the cell surface of the slow growing clones C6BP2-1, -4 and -9 is IGFBP-5.

7.4.6 Effect of exogenous IGF-I on the growth of the clones

To test whether a change in the balance between IGF-I and IGFBPs would alter the growth of clones, the wild type C6, and clones C6BP2-1 and -12 were analyzed in medium containing 1% FBS with or without the daily

Figure 7.6 Ligand blot analysis of CM from clones C6BP2-1, -4, and -12 (lanes 1-3) and FRTL-5 (lane 4), a rat thyroid cell line which secretes predominantly IGFBP-5. The 29 kDa IGFBP secreted by clones C6BP2-1, -4, but not C6BP2-12, comigrates with IGFBP-5 secreted by FRTL-5 cells.

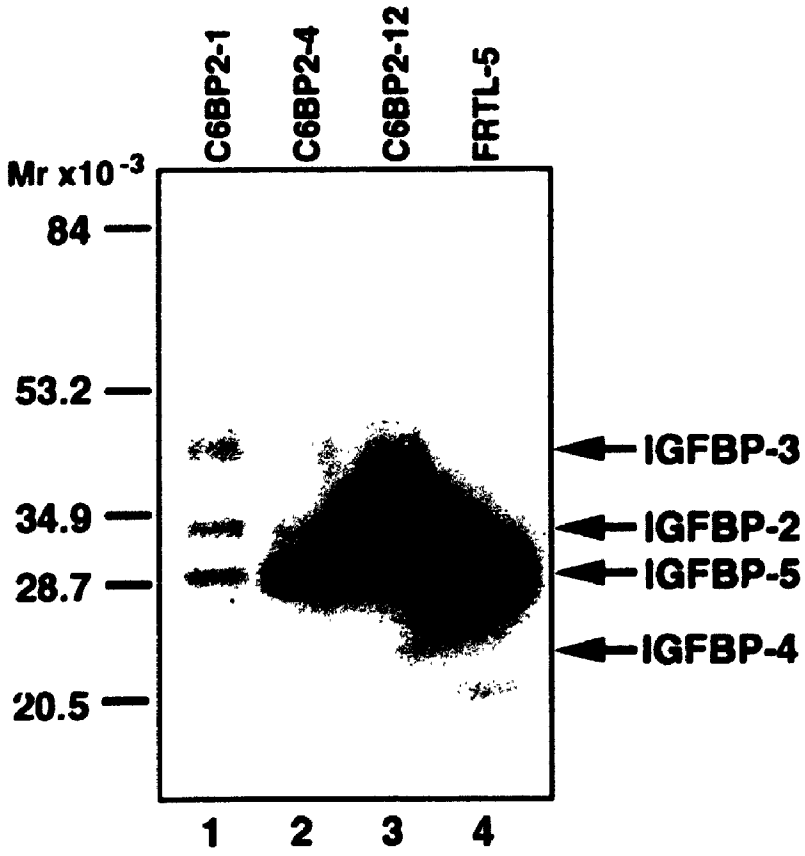
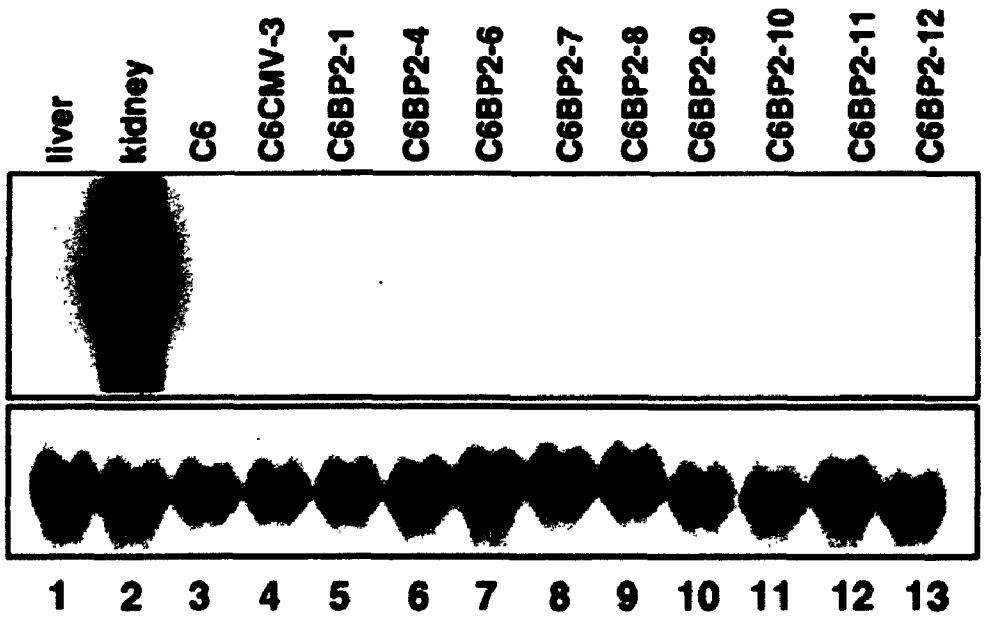

C6BP2-1
C6BP2-4
C6BP2-12
FRTL-5
Mr x10-3
84
53.2
34.9
28.7
20.5
IGFBP-3
IGFBP-2
IGFBP-5
IGFBP-4
1
2
3
4

Figure 7.7. Northern blot analysis of total RNAs (40 µg/lane) from adult rat liver (lane 1), adult rat kidney (lane 2), C6 glioma (lane 3), pRc/CMV vector transfected control (lane 4), and pRc/CMV-oIGFBP-2 transfected clones (lanes 5-13) probed with [^{32}P]labeled cDNAs for rat IGFBP-5 (upper panel) and 18 S ribosomal RNA (lower panel). The 6.0 kb IGFBP-5 transcript was detected in kidney, and in very low abundance, in clones C6BP2-1 and -4. IGFBP-1 and IGFBP-6 mRNAs were not detected in the C6 cells nor any of the transfected clones (not shown).


liver
kidney
C6
C6CMV-3
C6BP2-1
C6BP2-4
C6BP2-6
C6BP2-7
C6BP2-8
C6BP2-9
C6BP2-10
C6BP2-11
C6BP2-12
1 2 3 4 5 6 7 8 9 10 11 12 13

addition of exogenous IGF-I (200 ng/ml) (Figure 7.8). C6 and C6BP2-12 grew at similar rates and were faster than C6BP2-1. Cell number at 72 h was significantly greater for both C6 and C6BP2-12 compared to C6BP2-1. Addition of IGF-I to both C6 and C6BP2-12 resulted in a significant increase in cell number at 72 h when compared to their cell number in 1% FBS alone. In contrast, addition of IGF-I to C6BP2-1 did not result in any change in cell number at 72 h when compared to 1% FBS alone.

7.4.7 Exogenous IGFBP-5 will associate with the cell surface of wild type C6 glioma cells

To determine whether exogenous purified IGFBP-5 could associate with the cell surface of the wild type C6 cells, human recombinant IGFBP-5 was incubated with monolayers of C6 cells and clone C6BP2-1 and subsequently cross-linked with [^{125}I]IGF-II (Figure 7.9). The expected 260 kDa type 2 receptor was observed in both C6 cells and clone C6BP2-1. The expected 41 kDa cell surface associated IGFBP was observed in clone C6BP2-1, but not C6 cells, without pre-incubation with IGFBP-5. When IGFBP-5 was pre-incubated for 24 h prior to cross-linking, a 41 kDa band was observed in C6 cells. Also, a smaller band of 38 kDa was observed on both the C6 cells and clone C6BP2-1 with pre-incubation of IGFBP-5.

Figure 7.8. The growth curves of C6 (●), C6BP2-1 (▲), and C6BP2-12 (■) in medium containing 1% FBS with (filled symbols) or without (open symbols) the daily addition of IGF-I (200 ng/ml). Statistically significant increases in cell number at 72 h with the addition of IGF-I compared to without for the same clones is designated by * and ‡, $p<0.05$.

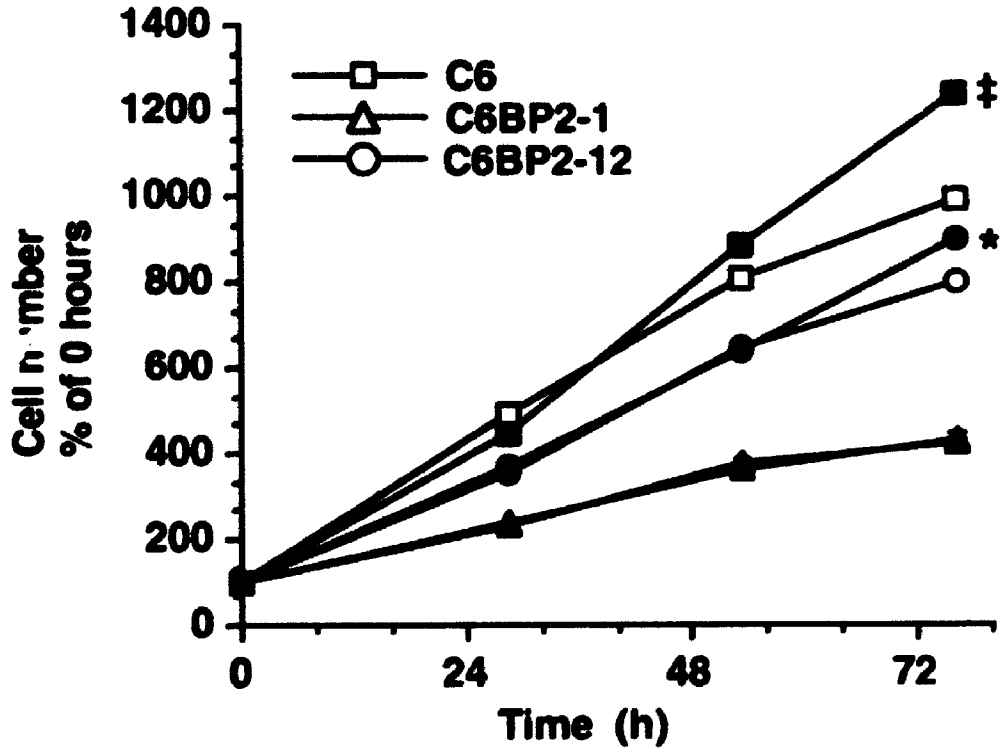
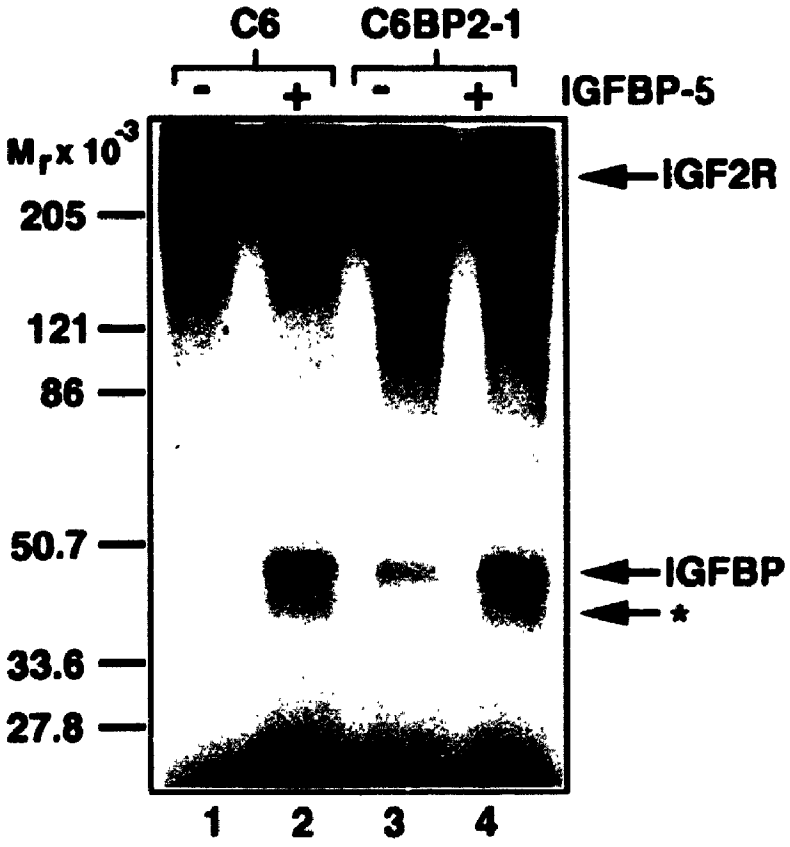

1400
1200
1000
800
600
400
200
0
Cell n 'mber
% of 0 hours
C6
C6BP2-1
C6BP2-12
‡
*
0
24
48
72
Time (h)

Figure 7.9 [^{125}I]IGF-II was crosslinked to the cell surface of C6 cells (lanes 1 and 2) and clone C6BP2-1 (lanes 3 and 4) following a 24 h pre-incubation with human recombinant IGFBP-5 (2.5 μg/ml; lanes 2 and 4) or SFM alone (lanes 1 and 3). The C6 cells showed a 41 kDa [^{125}I]IGF-II bound membrane complex that co-migrated with the cell surface associated IGFBP on clone C6BP2-1. An additional [^{125}I]IGF-II bound membrane complex of 39 kDa (*) was observed in both the C6 and clone C6BP2-1 cells pre-incubated with human recombinant IGFBP-5.


C6
C6BP2-1
- + - +
IGFBP-5
M_r x 10^{-3}
205
121
86
50.7
33.6
27.8
IGF2R
IGFBP
*
1 2 3 4

7.5 DISCUSSION

7.5.1 IGF receptor affinity was not altered in the clones transfected with the IGFBP-2 cDNA

Affinity cross-linking of [^{125}I]IGF-I and [^{125}I]IGF-II with competition from unlabeled IGF-I, IGF-II and insulin was used to examine the relative IGF receptor affinity in wild type C6 cells and clones C6CMV-3, C6BP2-1, -4, -9, -10, and -12. Each clone displayed a similar characteristic binding competition profile for both the type 1 and type 2 IGF receptors. The cross-linking technique and the presence of the cell surface associated IGFBP on some of the clones does not allow for an accurate quantification of IGF receptor number or analysis of receptor affinity. A reduction in IGF receptor number could also account for the lack of growth response of C6BP2-1 to exogenous IGF-I (Pietrzkowski et al. 1992b, Ambrose et al. 1994, Resnicoff et al. 1994b).

7.5.2 The cell surface associated IGFBP was deduced to be IGFBP-5

Several lines of evidence support the identity of the cell surface associated IGFBP as IGFBP-5. Immunoprecipitation of [^{125}I]IGF-II cross-linked complexes by an antiserum against bovine IGFBP-2 determined that the IGFBP observed in the cross-linking assay was a smaller molecular size than [^{125}I]IGF-II-IGFBP-2 cross-linked complexes detected in the conditioned media of the transfected clones. The RGD containing peptide failed to inhibit the association of the cell surface IGFBP further suggesting that its identity was not IGFBP-2. High concentrations of heparin did inhibit the cross-linking of [^{125}I]IGF-II to the cell surface associated IGFBP suggesting that the IGFBP may have a heparin binding domain (Hodgkinson et al. 1994, Booth et al. 1994).

More direct evidence suggesting that the cell surface associated IGFBP was IGFBP-5, came from the results of Northern and ligand blot analyses. The 29 kDa IGFBP, detected only in the conditioned medium of clones C6BP2-1, -4, and -9 which exhibited the cell surface associated IGFBP, co-migrated with the previously characterized IGFBP-5 secreted into the conditioned medium of FRTL-5 cells (Backeljauw et al. 1993). Using 40 ug of total RNA per lane, a 6.0 kb IGFBP-5 mRNA transcript was detected in clones C6BP2-1, and -4 further suggesting that the cell surface associated IGFBP was IGFBP-5. Three different IGFBP-5 antiserum did not recognize IGFBP-5 in the CM from FRTL-5 cells and therefore we were unable to immunologically characterize this IGFBP.

7.5.3 Cell surface associated IGFBP-5 and its relationship to cell growth

Although the variable expression of IGF-I among the different clones could account for the differences in growth, we have observed that different growth patterns are associated with changes in the level of IGF-I and IGFBPs, and IGFBP cellular localization. Consistent with the observation of concomitant increased expression of IGF-I and IGFBP-2, is the hypothesis that IGFBP-2 is acting as a competitive inhibitor for IGF-I binding to its receptor. When changes in the balance between IGFBP-2 to IGF-I occurred, as in those clones with IGFBP-2 overexpression and IGF-I underexpression, growth rates were significantly reduced. Alternatively, the association of IGFBP-5 with the cell surface of the slow growing clones, which displays a preferential affinity for IGFs compared to the receptors, may be the primary determinant for their slow growth. The observation that IGF-I further stimulated the growth of C6 and C6BP2-12, but not C6BP2-1, supports this last hypothesis that cell surface IGFBP-5 is acting to inhibit the growth of these clones by preventing the

interaction of IGF-I with its receptor. Similar concomitant changes in IGF-I and IGFBP-5 have been observed in fibroblasts transformed with Simian Virus 40 (Reeve et al. 1995). Transformation by SV40 not only increased expression of the IGF-I gene, but also resulted in a loss of cell surface associated IGFBP-5. In untransformed cells, IGF-I binding was principally to cell surface associated IGFBP-5 which inhibited its interaction with the type 1 receptor.

7.6 CONCLUSION

The exact mechanism(s) controlling the growth of the various C6BP2 clones remains to be determined, however, it is clear that the interactions between IGF-I and IGFBPs remain an important determinant of their growth. Whether IGFBP-2 and/or IGFBP-5 have unique role in the growth of glial cells remains to be clarified. This study suggests that glial cells may have a limited capacity to modulate various components of the IGF system in order to maintain normal growth as evidenced by the concomitant upregulation of IGF-I and downregulation of endogenous IGFBPs, as well as production of IGFBPs with specific characteristics. It also underlines the importance of examining all components of the IGF system before formulating conclusions on their role in cellular growth. These studies suggest that growth of glial cells is regulated by a coordinated interaction between IGF-I and IGFBPs, and specific properties (i.e.. cell surface association) of the latter.

CHAPTER EIGHT

GENERAL DISCUSSION

8.1 SUMMARY

Astroglia synthesize IGFs, IGFBP-2 and IGFBP-3 which play a role in the regulation of their growth in an autocrine or paracrine manner. Astroglial IGFBPs are regulated by their endogenous ligands, IGF-I and IGF-II, and by insulin. IGF-I, IGF-II and insulin increased both IGFBP-2 and IGFBP-3 protein and stable mRNA levels. The time course of IGF-I induction of IGFBP-2 and IGFBP-3 mRNAs occurred rapidly by 6 h. For IGFBP-2, stable mRNA levels declined by 12 h and increased again by 24 h. IGFBP-3, however, maintained peak levels until 12 h and subsequently declined to below control levels by 18 and 24 h.

Astroglial IGFBPs were also regulated by EGF, TGF-α, acidic FGF and basic FGF. EGF and TGF-α increased both IGFBP-2 and IGFBP-3 protein levels detected in the conditioned media, however, they had differential effects on stable mRNA levels. EGF, but not TGF-α, increased IGFBP-2 and IGFBP-3 stable mRNA levels. EGF induced IGFBP-2 mRNA levels in a time course similar to IGF-I. EGF induced IGFBP-3 mRNA levels rapidly ... increases were observed by 6 h which remained steady until 12 h and subsequently increased to a maximum at 24 h. Acidic FGF and basic FGF similarly increased IGFBP-2 and IGFBP-3 protein and stable mRNA levels however, basic FGF was more potent than acidic FGF. The time course of IGFBP-2 and IGFBP-3 mRNA induction was similar for both acidic FGF and basic FGF with maximum levels occurring by 6 h.

Primary astroglial cells with regulated growth express abundant connexin43 while C6 cells derived from a rat glioma have reduce connexin43 expression. Different clones of C6 glial cells with variable expression of a transfected connexin43 cDNA and differing growth patterns, secreted different

profiles of IGFBPs and IGF-I. The highest overexpresser of connexin43 (Cx43-13) which had increased intercellular communication and a reduced proliferative capacity, upregulated the expression of IGFBP-4 and downregulated the expression of IGFBP-3 and IGF-I compared to the wild type C6 cells. The intermediate expressers which also displayed increased intercellular communication and a reduced proliferative capacity, although to a lesser extent than the highest overexpresser, downregulated IGF-I expression but IGFBP-3 and IGFBF-4 were not affected. The clone Cx43-13 was able to respond mitogenically to exogenously added IGFs in contrast to the wild type C6 cells. In addition, exogenously added IGFs were able to reverse the growth inhibitory effect of the conditioned media of clone Cx43-13.

To examine the biological role of IGFBP-2 in the growth of astroglial cells, an ovine IGFBP-2 cDNA was overexpressed in C6 glioma cells. Changes in the growth rate of the clones from the wild type C6 cells did not correspond to the level of over-expression of IGFBP-2. Clones overexpressing very high levels of the IGFBP-2 transgene, also expressed correspondingly high levels of IGF-I mRNA and peptide, and had similar growth rates compared to the wild type C6 cells. In contrast, clones overexpressing moderate levels of the IGFBP-2 transgene, with no increase in IGF-I mRNA and peptide, and had the cell surface associated IGFBP-5, had significantly reduced growth rates compared to the wild type C6 cells. Those clones which had the cell surface associated IGFBP-5 did not exhibit an increase in cellular growth in response to exogenous IGF-I.

8.2 CONCLUSIONS

The regulation of IGFBPs by their endogenous ligands (IGFs) suggests that the astroglial cell regulates the level of IGFBPs to maintain an equilibrium between IGF and IGFBP required for homeostasis within this tissue system. The presence of increased IGF levels in the extracellular *milieu* would result in the increased activation of IGFBP genes and an increase in the production and secretion of IGFBPs. The increased levels of IGFBPs would sequester excess IGFs and thus modulate their biological activity upon the cells. This feedback mechanism would be important in maintaining cellular homeostasis and regulated growth which is crucial to the developmental process.

The greater increase in IGFBP-3 protein compared to IGFBP-2 by IGFs, may reflect release of cell surface associated IGFBP-3 in addition to the increased IGFBP-3 mRNA levels stimulated by [illegible]t growth factors. This phenomenon has been observed in other cell systems as discussed previously (Conover et al. 1991, Conover 1992). Alternatively, there may be differences in the translatability of IGFBP-2 and IGFBP-3 mRNAs or other post-translational mechanisms such as intracellular transport, post-translational modification, and secretion.

For all growth factors examined, the detected increase in IGFBP-2 protein was less in magnitude compared to the increase in IGFBP-3. The greatest fold increase in IGFBP-2 protein occurred with its endogenous ligands, IGF-I and IGF-II. This apparent stricter regulation of IGFBP-2 compared to IGFBP-3, suggests that IGFBP-2 may have more direct biological effects than IGFBP-3 in this cell system. Indeed, IGFBP-2 expression was correlated with different growth phenotypes. Primary astroglial cells, which

demonstrate regulated growth, synthesize high levels of IGFBP-2, while C6 glioma cells, with tumourigenic growth, express very low levels of IGFBP-2. In addition, some of the clones of C6 cells overexpressing IGFBP-2 exhibited a reduction in their growth rate.

The greater responsivity of IGFBP-3 may also reflect its biological role in this cell system. Changes in IGFBP-3 expression was observed both in the connexin43 overexpressing clones and the IGFBP-2 overexpressing clones, however, they did not correlate with observed changes in the growth phenotype, suggesting that IGFBP-3 is less important in maintaining 'normal' growth rates. The greater responsiveness of IGFBP-3 in response to various growth factor stimuli may be important in conditions of brain injury. Reactive astroglia express a number of growth factors during injury which are thought to stimulate their own proliferation and aid in axonal regeneration (Steward 1989). IGFBP-3 may participate in their increased proliferation in an autocrine manner. IGFBP-3 may also be important in potentiating the biological actions of IGF-I on neuronal sprouting, regeneration, and survival (Aizenman and deVellis 1987, Caroni and Grandes 1990, Ishii et al. 1994, Recio-Pinto et al. 1984, 1986, 1988, Wang et al. 1992). IGFBP-3 has been shown to accelerate IGF-I stimulated wound healing in other cell systems (Sommer et al. 1991, Hamon et al. 1993).

The results from the connexin43 expressing C6 clones and the IGFBP-2 expressing C6 clones support a role for IGF-I in regulating the proliferation of astroglial cells. The degree of downregulation of IGF-I gene expression corresponded with the level of overexpression of the connexin43 cDNA. This contrasted with the observed changes in expression of IGFBP-3 and IGFBP-4 suggesting that IGF-I may have a more important role in cell proliferation compared to IGFBP-3 and IGFBP-4. This effect was also observed with the

IGFBP-2 transfected C6 clones where a decrease in cellular growth corresponded to a decrease in IGF-I expression regardless of the expression of IGFBP-3 and IGFBP-4. These results agree with others demonstrating that IGF-I is an important regulator of C6 cell growth (Trojan et al. 1992). Similar studies in other cell types have demonstrated that loss of expression of IGF-I leads to reduced growth and tumourigenicity (Pietrzkowski et al. 1992b, Ambrose et al. 1994).

However, the changes in growth observed with the IGFBP-2 overexpressing clones, corresponded to changes in IGF-I, IGFBP-2 expression, and IGFBP-5 expression and cellular localization. These findings suggested that growth of astroglial cells was regulated by a coordinated interaction between IGF-I and IGFBPs, and specific properties (i.e. cell surface association) of the latter. The role of cell surface associated IGFBP-5 in modulating growth of astroglial cells remains to be defined.

This study demonstrates that glial cells may have a limited capacity to modulate various components of the IGF system in order to maintain normal growth as evidenced by the concomitant upregulation of IGF-I. The level of expression of IGFBP-2 tended to correlate with the level of upregulation of IGF-I in those clones which maintained a wild type growth rate suggesting that IGF-I expression may be regulated by IGFBP-2. The increased levels of IGFBP-2 could bind IGF-I present in the extracellular milieu and result in an increased production of IGF-I by the cell. This supports the hypothesis that astroglial cells regulate the expression of IGF-I and IGFBP-2 to maintain an appropriate equilibrium required for homeostasis within this tissue system.

8.3 FUTURE STUDIES

To fully characterize the regulation of astroglial IGFBPs, additional levels of regulation need to be examined including, the mechanisms of IGFBP secretion, release of cell surface associated IGFBPs and stability of IGFBPs in the conditioned media. In addition, determining whether the increased stable IGFBP mRNA levels reflect an increase in rate of transcription or an increase in mRNA stability will be required to more completely understand the mechanisms involved in the regulation of astroglial IGFBPs.

To fully understand the interaction of EGF, TGF-α, acidic FGF and basic FGF with the IGF system, their regulation of IGFs and IGF receptors will need to be determined. In addition, the expression of the various components of the EGF family and FGF family of growth factors and their effects, both alone and in combination with IGFs, on astroglial growth will be required to understand the interaction of these growth factors with the IGF system.

The role of cell to cell contact and the mechanisms underlying the changes in growth factor regulation need to be defined in order to elucidate the interactions between intercellular communication, growth factors and regulation of cell growth in both normal and tumourigenic cells.

Defining the mechanism(s) that control the growth of the various C6BP2 clones remains to be determined. Further studies employing purified IGFBPs and IGFs, to manipulate the balance of IGF to IGFBPs, will help to clarify the interaction between IGFs and IGFBPs in regulating the growth of astroglial cells and determining whether IGFBP-2 and/or IGFBP-5 have unique roles.

APPENDIX I

Saline I

0.139 M sodium chloride
5.4 mM potassium chloride
1.1 mM sodium phosphate dibasic
1.1 mM potassium phosphate monobasic
22 mM dextrose
in dd H_20

Phosphate buffered saline (PBS)

2.9 mM sodium phosphate monobasic
19.4 mM sodium phosphate dibasic
0.3 M sodium chloride
in dd H_20

Antibody diluting solution

1% (w/v) BSA
0.02% sodium azide
in PBS

1x Laemmli Buffer

2% SDS
10% glycerol
60 mM Tris pH 6.8
0.001% bromophenol blue
100 mM DTT
in dd H_20

Tris buffered saline (TBS)

150 mM NaCl
50 mM Tris-HCl, pH 8.0
in dd H_20

TTBS

0.05% Tween 20 in TBS

Guanidine thiocyanate solution

4 M guanidine thiocyanate
25 mM sodium citrate
17 mM N-lauroyl sarcosine
0.7% B-mercaptoethanol, pH 7.0
in dd H_20

Cesium chloride solution

5.7 M cesium chloride
0.1 M EDTA, pH 7.4
in dd H_20

20x Northern buffer

0.4 M MOPS
100 mM sodium acetate
10 mM EDTA, pH 7.4
in dd H_20

20x SSC

3 M sodium chloride
0.3 M sodium citrate, pH 7.0
in dd H_20

TE buffer pH 7.4

10 mM Tris-HCl pH 7.4
1 mM EDTA pH 8.0
in dd H_20

Northern hybridization buffer

50% formamide
5x SSPE
7% SDS
5 µg/ml denatured salmon sperm DNA
in dd H_20

5x SSPE

0.75 M NaCl
44 mM $NaH_2PO_4.2H_2O$
5mM EDTA pH 7.4
in dd H_20

HEPES binding buffer

0.1 M HEPES
7.75 mM sodium phosphate dibasic
2.25 mM sodium phosphate monobasic
0.1% BSA
pH 7.4
in dd H_20

Cross-linking buffer

0.1 M HEPES
7.75 mM sodium phosphate dibasic
2.25 mM sodium phosphate monobasic
pH 7.4
in dd H_20

Membrane homogenization buffer

20 mM TRIS, pH 7.5
0.33 M sucrose
2 mM EDTA
0.5 mM EGTA
1% Nonidet P-40
2 mM PMSF
0.3% aprotinin
in dd H_20

In situ hybridization buffer

50% formamide
0.3 M NaCl
20 mM Tris pH 8.0
1 mM EDTA
1x Denhardt's solution from 50x stock
500 µg/ml yeast tRNA
100 µg/ml denatured salmon sperm DNA
10% dextran sulfate
0.1% SDS
100 mM DTT
in dd H_20

50x Denhardt's solution

1% (w/v) Ficoll
1% (w/v) polyvinylpyrrolidone
1% (w/v) BSA)
in dd H_20

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